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Modulatory interventions in post-natal hippocampal neurogenesis: structural and functional implications

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"If the doors of perception were cleansed
everything would appear to man as it is, infinite".
William Blake,
The Marriage of Heaven and Hell, 1793.

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To my parents, for their character and strength of will.

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To my friends, for all the support and energy.

Modulatory interventions in post-natal hippocampal neurogenesis: structural and functional implications

Abstract

Post-natal neurogenesis is one of the most interesting brain events disclosed in the last century, with its modulation promising a new approach to the treatment of some central nervous system (CNS) pathologies. Despite the expectations, its clinical application is not yet a reality, mainly because the information concerning its function and regulatory mechanisms is still insufficient.

In order to better understand the mechanisms that regulate adult neurogenesis, we decided to assess the topographic distribution of hippocampal neurogenesis and apoptosis in basal conditions (Chapter 2.1). We showed that neither neurogenesis nor apoptosis occur homogeneously within the dentate gyrus as each of these events display different gradients. Therefore, the existence of specific gradients of cell birth/death in the adult hippocampal formation is a factor that must be taken into account whenever studying these events because the function of the hippocampal formation differs from one sub-region to the other. Interestingly, we reported for the first time a higher apoptotic density in the left hippocampal dentate gyrus.

Our next question concerned the assessment of how both events (cell birth/death) were related to depressive-like behaviour (Chapter 2.2). To address these goals we used a chronic-mild-stress protocol (CMS) to induce a depressive-like behaviour in rats (assessed in the forced-swimming test (FST)). Besides being a factor that predisposes to depression, stress exposure also influences hippocampal neurogenesis and structure. In accordance, we observed a decrease of hippocampal cell turnover after CMS as a result of an increase in apoptosis and a reduction of hippocampal neurogenesis.

One of the hallmarks of stress response is the raise of corticosteroids levels. Corticosteroids can bind to the mineralocorticoid (MR) and the glucocorticoid receptor (GR). The differential activation of corticosteroid receptors is considered to be crucial in determining the effects of these hormones in the brain. Thus, in Chapter 2.3 we assessed the impact of different corticosteroids receptor activation in hippocampal cell turnover. All experimental paradigms triggering imbalances in corticosteroid milieu induced depressive-like behaviour, but the analysis of the final cell balance (proliferation – apoptosis) revealed that treatment with a selective GR agonist (dexamethasone - Dex) produced stronger deleterious effects than those observed after the use of a mixed ligand of corticosteroid receptors (corticosterone - Cort), suggesting a protective role of MR. In further support of the neuroprotective action triggered by MR activation, adrenal removal (ADX) also induced a decrease in final hippocampal cell balance, because the raise of apoptosis outweighed that of

neurogenesis. These data show that depressive-like behaviour can occur despite increased neurogenesis, highlighting the advantage of an analysis of global cell turnover.

In order to establish some of the molecular pathways through which stress/corticosteroids could be acting, we analysed the expression of the enzyme GSK-3β, of β-catenin (a protein of the Wnt pathway that is a target of GSK-3β) and of VEGF (an angiogenic factor that is regulated by β-catenin) (Chapter 2.2; 2.3 and 2.4). Furthermore, two other down-stream targets, synapsin-I (a pre-synaptic protein), and the anti-apoptotic BAG-1 were also analyzed to establish correlations of synaptic plasticity and cell fate, respectively (Chapter 2.2; 2.3 and 2.4). We observed that stress increased GSK-3β and decreased the expression of synapsin-I, BAG-1, β-catenin and VEGF; these stress-actions were largely mediated by GR activation, since Dex treatment reproduced these effects. However it must be also notice that corticosteroid receptors unoccupancy (through ADX) also produced a decrease of BAG-1 and synapsin-I. These results indicate that the behavioural and hippocampal cell turnover effects induced by stress/corticosteroids occur through the modulation of pathways of neurogenesis, angiogenesis, apoptosis and synaptic plasticity.

A final aim of this work was the search for pharmacologic modulators of post-natal hippocampal cell turnover. Lithium, a mood stabilizer with antidepressant effects, is used in clinical practice for decades and was previously shown to increase neurogenesis and decrease apoptosis. In order to discriminate lithium effects in hippocampal functionality, and the molecular pathways through which it may act, we analyzed its actions in depressive-like behaviour and cell fate. We observed that lithium administration to stress-free animals increased final-cell balance but also triggered signs of depressive-like behavior. However, when concomitantly administrated with stress or corticosteroids, lithium administration prevented the behavioural, plastic and molecular effects induced by corticosteroid manipulations. These data highlighted some of the mechanisms through which lithium can modulate cell fate and possibly induce its therapeutic actions.

In conclusion, the variations in hippocampal cell turnover constitute a better biomarker of hippocampal formation's structural and functional integrity than neurogenesis *per se*. Stress, or imbalance of corticosteroids milieu, affect hippocampal cell turnover; these actions are at least partially mediated by modulation of GSK-3β. In accordance, administration of lithium (a mood stabilizer that inhibits GSK-3β) prevented the insult-induced effects. We hope that these results may contribute to the comprehension of the relevance of hippocampal cell turnover in basal and stress-associated pathological conditions, and also to elucidate some mechanisms through which cell fate modulators might act.

Intervenções Moduladoras da neurogénese hipocampal pós-natal: implicações estruturais e funcionais

Resumo

A neurogénese hipocampal pós-natal é um dos mais interessantes eventos cerebrais descobertos no último século, com a sua modelação prometendo uma nova abordagem para o tratamento de algumas patologias do sistema nervoso central (SNC). Contudo, apesar das expectativas, a sua aplicação clínica ainda não é uma realidade, principalmente devido à insuficiente informação sobre a sua função e seus mecanismos reguladores.

De modo a compreender os mecanismos que regulam a neurogénese no adulto, decidimos avaliar a distribuição topográfica basal da neurogénese e apoptose na circunvolução denteada da formação hipocampal (Capitulo 2.1). Mostramos que nenhum destes eventos ocorre de forma homogénea devido ao facto de ambos apresentarem diferentes gradientes. Assim, a ocorrência de gradientes hipocampais específicos de nascimento e morte celulares, é um factor que deve ser tido em consideração aquando do estudo destes eventos, pois a função da formação hipocampal muda de acordo com a sub-região. É de relevar que mostramos pela primeira vez a existência de uma maior densidade apoptótica na circunvolução denteada esquerda.

A nossa próxima questão centrou-se na análise do modo como o nascimento e morte celulares estão relacionadas com o comportamento semelhante ao depressivo (Capitulo 2.2). Para tal foi usado um protocolo de *chronic-mild stress* (CMS) de modo a induzir um comportamento semelhante ao depressivo (avaliado no *forced-swimming test* (FST)). Para além de ser um factor predisponente para a depressão, a exposição ao stress também influencia a neurogénese e estrutura da formação hipocampal. Em concordância, nós observamos uma diminuição do balanço celular (proliferação – apoptose) na formação hipocampal depois do CMS, devido a um aumento da apoptose e redução da neurogénese.

Um dos marcos da resposta ao stress é o aumento dos níveis de corticosteróides. Os corticosteróides podem ligar-se ao receptor mineralocorticoide (MR) ou ao glucocorticóide (GR). A activação diferencial dos receptores dos corticosteróides é considerada crucial para os efeitos destas hormonas no cérebro. Assim, no Capítulo 2.3 estudamos o impacto da activação dos diferentes receptores dos corticosteróides no balanço celular da circunvolução denteada da formação hipocampal. Todos os paradigmas experimentais que induziram uma alteração dos níveis de corticosteróides levaram um comportamento semelhante ao depressivo. Contudo, a análise do balanço celular revelou que o tratamento com um agonista selectivo dos GR (dexametasona- Dex) induziu maiores efeitos deletérios do que o tratamento com um ligando misto dos receptores dos corticóides (corticosterona – Cort), sugerindo um papel protector dos MR. Enfatizando a acção neuroprotectora da activação dos MR, a

remoção das supra-renais (ADX) também levou a uma diminuição do balaço celular hipocampal, pois o aumento da apoptose foi superior ao aumento da neurogénese. Estes dados mostram que um comportamento similar ao depressivo pode ocorrer apesar de um aumento da neurogénese, o que realça a vantagem de uma análise global do balanço celular.

De modo a estabelecer algumas das vias moleculares através das quais o stress/corticosteróides poderiam estar a actuar, analisamos a expressão da enzima GSK-3β, da β-catenina (uma proteína da via Wnt e que é um alvo da GSK-3β) e do VEGF (um factor angiogénico que é regulado pela β-catenina) (Capítulos 2.2, 2.3 e 2.4). Adicionalmente, para estabelecer correlações de plasticidade sináptica e destino celular, outros dois alvos da GSK-3β foram analisados: respectivamente, a synapsina-I (uma proteína pré-sinaptica) e a proteína anti-apoptótica BAG-1 (Zapítulos 2.2, 2.3 e 2.4). Observamos que o stress aumentou a expressão da GSK-3β e diminui a de sinapsina-I, BAG-1, β-catenina e VEGF; estas acções do stress foram grandemente mediadas pela activação dos GR, pois a Dex reproduziu estes efeitos. Contudo, também deve ser tido em consideração que a não ocupação dos receptores dos corticosteróides (ADX) também diminui a expressão de BAG-1 e sinapsina-I. Estes resultados indicam que os efeitos do stress/corticosteróides no comportamento e balanço celular ocorrem pela modulação das vias da neurogénese, angiogénese, apoptose e plasticidade sináptica.

Um objectivo final deste trabalho foi o estudo de moduladores farmacológicos do balanço hipocampal pós-natal. Previamente foi reportado que o lítio, um estabilizador do humor com efeitos antidepressivos usado há décadas na prática clínica, aumenta a neurogénese e diminui a apoptose. Para a discriminar os efeitos do lítio na funcionalidade hipocampal e as vias moleculares por onde ele poderá actuar, analisamos as suas acções no comportamento similar ao depressivo e no destino celular. Foi observado que a administração de lítio a animais não stressados aumenta a balanço celular mas também despoleta sinais de comportamento similar ao depressivo. Contudo, quando concomitantemente administrado com o stress ou corticosteróides, o lítio previne efeitos comportamentais, plásticos e celulares induzidos destes insultos. Estes dados revelam alguns dos mecanismos através dos quais o lítio pode modular o destino celular e também induzir as suas acções terapêuticas.

Como conclusão, as variações do balanço celular hipocampal constituem um melhor biomarcador da integridade estrutural e funcional da formação hipocampal do que a neurogénese por si. O stress ou alterações dos níveis de corticoesteróides afectam o balanço celular hipocampal; estas acções são, pelo menos parcialmente, mediadas pela modulação da GSK-3β. Concordantemente, administração de lítio (um estabilizador do humor que inibe a GSK-3β) previne os efeitos induzidos por estes insultos. Esperamos que resultados aqui apresentados possam contribuir para a compreensão da relevância do balanço celular hipocampal em condições basais ou patológicas associadas ao stress, e também elucidar alguns mecanismos pelos quais os moduladores do destino celular possam actuar.

Contents

Abreviations List

ACTH	Adrenocorticotropic hormone
ADX	Adrenalectomy
APC	Adenomatous polyposis coli protein
BAG-1	B-cell-CLL/lymphoma2-associated athanonege
Bax	B-cell CLL/lymphoma 2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
BDNF	Brain derived neurotrophic factor
BrdU	Bromodepxyuridine
CA	Cornu Ammonis
CdK	Cyclin-dependent kinases
Cort	Corticosterone
CREB	Cyclic AMP response element biding protein
CRF	Corticotropin releasing factor
DAG	Diacylglycerol
DCX	Doublecortin
Dex	Dexamethasone
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FGF	Fibroblast growth factor
GCL	Granule cell layer
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
GSK-3	Glycogen synthase kinase-3
Hi	Hilus
HPA	hypothalamus-pituitary-adrenal
HRE	Hormone response element
HSF-1	Heat shock factor-1
Hsp-70	Heat shock protein-70
IGF	Insulin growth factor

IMPase	Inositol monophosphate phosphatase
IP_2	Inositol-1, 4, 5-triphosphate
IPPase	Inositol polyphosphate 1-phosphatase
IR	Insulin receptor
LEF	Lymphoid enhancer factor
Li	Lithium
LTP	Long term potentiation
MAP kinase	Mitogen-activated protein kinase
ML	Molecular layer
MR	Mineralocorticoid receptor
NeuN	Neuronal nuclei protein
NOS	Reactive nitrogen species
NT	Neurotrophin
PKC	Protein kinase C
PI	Phosphoinositol
PiP_2	phosphoinositide 4,5-biphosphate
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PVN	Paraventricular nucleus of the hypothalamus
ROS	Reactive oxygen species
SGZ	Subgranular zone
SVZ	Subventricular zone
TCF	T-cell factor
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1. Introduction

1.1 A brief historical note

Adult neurogenesis is one of the latest but also one of the most exciting discoveries in the history of neurosciences. For long it was believed that neurogenesis, which occurred in embryogenesis, was no longer present in the postnatal brain. One of the most famous sentences supporting this view is attributed to Ramon y Cajal (1928): "In the adult centres, the nerve paths are something fixed and immutable: everything may die, nothing may be regenerated". Although there were already studies indicating that the brain may not be completely immutable in terms of cell genesis, this axiom would haunt neuroscientists to present days. The first report on cell division in the adult brain was published in 1912 by Ezra Allen, who demonstrated mitotic figures in the lateral ventricles walls. Almost four decades later Messier and colleagues showed, using tritiated thymidine which is incorporated by DNA in dividing cells, that labelling occurred in the subependymal zone of the lateral ventricles (Messier et al., 1958).

Despite all the previous studies, the general awareness of adult neurogenesis occurred only in the 60's with the works of Joseph Altman in the rat hippocampus. After two reports where Altman showed the presence of cells labelled with tritiated thymidine around a lesion site (Altman 1962) and in the cortex, (Altman, 1963), in 1965 he published with Gopal D. Das a study which is generally considered a milestone in the neurogenesis field. In that study, Altman and Das demonstrated that: i) neurogenesis occurred in the granule cell layer of the hippocampal dentate gyrus; ii) these newly born cells survived at least for two months and iii) although there was a conspicuous decline with age, neurogenesis could still be found in rats with eight months of age. At the same time, they also described the presence of smaller stained cells with dark nuclei, and so they put forth the proposal that these might be precursor cells (Altman and Das 1965a). In the same year, Altman and Das reported that newly born cells in the subependymal zone migrate to the olfactory bulb and differentiate into neurons (Altman and Das 1965b). This migration or "rostral migratory stream" was later (1969) described in detail by Altman.

A decade would pass after Altman's striking discoveries until another study revolutionized the field. In 1977, Kaplan and Hinds proved the neuronal nature of newly born cells in the hippocampus and olfactory bulb of rat's brain through the use of electron microscopy. The

following step was achieved by Fernando Nottebom by showing that tritiated thymidine labelled cells were electrophysiological active (Paton and Nottebhom, 1984).

The last decade of the XXth century was to the field one of the most productive. Some years after Nottebhom's work, Elizabeth Gould showed that adult hippocampal neurogenesis could be regulated, in this case down-regulated, by stress and corticosteroids (Gould et al., 1991; Cameron and Gould, 1994). Frank Corotto replaced the labelling of proliferating cells with tritiated thymidine by bromedeoxyuridine (BrdU), an analogue of thymidine, which is incorporated in the S phase of the cell cycle; this made possible the use of immunohistochemistry to study adult neurogenesis (Corotto et al., 1993). By the end of the decade, neurogenesis studies went even further with the reports of neuronal proliferation in primates (Gould et al., 1997) and in human adult hippocampus (Eriksson et al., 1998).

From the last major events in the neurogenesis history it possible to highlight the studies of Bettina Seri, from Alvarez-Buylla group, and Henriette van Praag, who worked with Alfred Gage. Seri and colleagues elegantly reported that the precursor cells of newly born neurons were astrocytes (Seri et al., 2001). Henriette van Praag (2002), using retrovirus with green fluorescent protein, showed that newly born neurons in the hippocampal dentate gyrus of rat brain develop into functional granule cells (van Praag et al., 2002), which indicated that not only in birds but also in mammals, newly born neurons became integrated in the system.

1.2 The structure of hippocampal formation

The hippocampal formation, together with the amygdala, septal nuclei, hypothalamus, habenula, anterior thalamic nuclei and part of the basal ganglia, constitute the limbic system (MacLean 1952) (Fig. 1). Briefly, the limbic system is responsible for the integration of sensorial and emotional information, visceral activity control, temporal and spatial orientation, learning and memory.

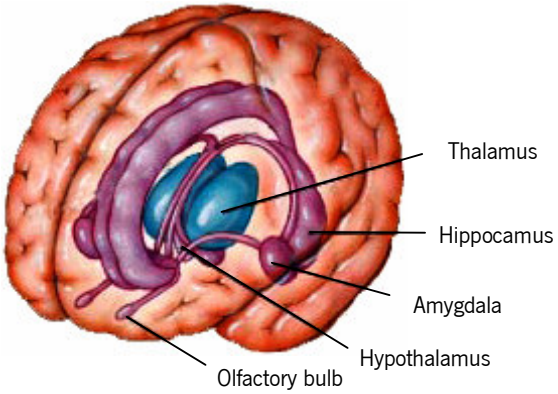

Fig. 1: Schematic representation of some structures of the human limbic system.

The hippocampal formation[1], a central component of the controversial concept of the limbic system, received its name due to its similarity, in a coronal section, to the outline of a seahorse (Arantius 1587). After being removed from the ventricle, the hippocampus resembles the horn of a ram, and Garengeot, in 1742, name it cornu ammonis (Ammon's horn) in honour to the Egyptian supreme god, Ammon.

From an evolutionary point of view, the hippocampal formation can be considered as being part of the "old cortex", the arquicortex, which is typically composed of three cell layers: a deep plexiform layer, a main neuronal band and an external fiber layer. In the dentate gyrus (or fascia dentata) the main cell type is the granule cell, forming the granule cell layer (GCL), which has a "V" or "U" shape. Its shape made it possible to distinguish between the portion of the layer near CA1 field (called suprapyramidal blade) and the opposite (called infrapyramidal blade); the region where the two blades unite is named the crest. The dendrites of granule cells extend to the overlying molecular layer (ML) where they receive the major afferent projections (namely from the entorhinal cortex). The plexiform layer, or hilus (Hl), resides inner to the granule cell layer.

The cornu ammonis, as the dentate gyrus, has a band of pyramidal cells. External to this band, there is the stratum oriens, the area where the basal dendrites of the pyramidal cells extend. Internal to the pyramidal cells band, the plexiform layer is subdivided in three strata: lucidum[2]

[1] There is a distinction between hippocampus and hippocampal formation, even though several authors do not make such distinction. The hippocampal formation contains the hippocampus proper and the dentate gyrus. For the purpose of this thesis the hippocampal formation will be considered as being constituted by the dentate gyrus, cornu ammonis and the subiculum.

(where the mossy fibers end), radiatum (where the Shaffer collaterals travel) and lacunosum-moleculare (where the perforant pathway passes). Lorente de Nó (1933) subdivided the cornu ammonis in CA1, CA2, CA3 and CA4, being this nomenclature still used.

The subiculum is constituted by three layers, i) a superficial molecular layer containing the apical dendrites ii) a medial pyramidal layer and iii) a deep polymorphic layer. The main internal hippocampal circuit, which connects the enthorinal cortex, the dentate gyrus, the cornu ammonis and the subiculum, is tri-synaptic, unidirectional and uses glutamate as its main neurotransmitter.

The major afferent projections to the dentate gyrus come from the entorhinal cortex by the perforant path, which establish synapses with the granule cells. These neurons project to the CA3 pyramidal cells through the mossy fibers. The pyramidal cells of CA3, via the Shaffer collateral pathway, connect with CA1 neurons, which project to the subiculum; the subiculum, in turn, projects to the enthorinal cortex, closing the circuit. The subiculum is the major efferent area of the hippocampus, projecting mainly to subcortical nuclei. The axons of the pyramidal cells of the subiculum constitute part of the alveus, which converge in the fimbria that subsequently integrates the fornix.

A characteristic of the adult hippocampal formation, namely the dentate gyrus, is its capacity to generate new neurons. Adult hippocampal neurogenesis occurs predominantly in a functional division of the GCL, the subgranular zone (SGZ) (Altman and Das 1965). The SGZ is generally considered as a 3-cell-body-wide zone in the inner side of the GCL, at the border between the GCL and Hi.

1.3 Adult Neurogenesis

In basal conditions, adult neurogenesis occurs mainly in two main areas of the brain: the subventricular zone of the lateral ventricles (SVZ) (Fig. 2) and the subgranular zone of the hippocampal dentate gyrus (SGZ) (Fig. 2). In rodents, during the first days of life there is also neuronal proliferation of cerebellar granule cells. However, in contrast to the SVZ and SGZ neurogenesis in the cerebellum terminates soon after birth. Despite the similarities in the

[2] This is only recognized in the CA3 region.

neurogenesis processes, the SVZ and SGZ present some differences: i) quantitatively SVZ neurogenesis largely outnumbers SGZ's; ii) newly born cells of SVZ migrate longer distances from their proliferation site to their final location (rostral migratory chain), when compared to SGZ's neuroblasts; and finally, iii) whereas SVZ neurogenesis originates new inhibitory interneurons of the olfactory bulb's granular cell layer and periglomerular region (Luskin 1993), SGZ produces new excitatory neurons of the granular cell layer of the hippocampal dentate gyrus (Ambrogini et al., 2004). This thesis will mainly focus in adult hippocampal neurogenesis.

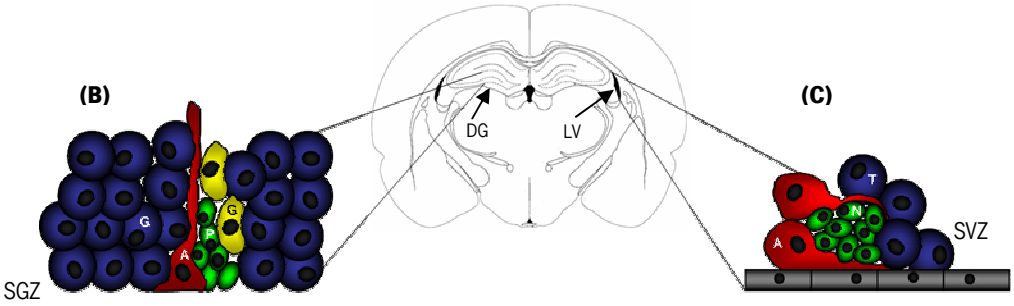

Fig. 2: (A) Schematic representation of an adult mouse brain coronal section. **(B)** Magnification of hippocampal dentate gyrus (DG) subgranular zone (SGZ) cytoarchitecture, including: astrocytes (A), which give raise to progenitor cells (P) that maturate into granule cells (yellow G cells). These cells integrate into the pre-existing dentate granule cell network (blue G cells). **(C)** Magnification of showing lateral ventricle (LV) subventricular zone (SVZ) cytoarchitecture, including: astrocytes (A), which are SVZ stem cells; T cells that are transit-amplifying cells derived from the B cells; T cells give rise to neuroblasts (N), which migrate to the olfactory bulb where they differentiate in interneurons.

The actual definition of adult neurogenesis comprises proliferation, migration, differentiation and integration of newly born neurons. Hippocampal neurogenesis occurs from progenitor cells with self-renewal limited capacity that are arranged in clusters near blood vessels; interestingly, the progenitor cells are attached by processes to this blood vessels (Palmer et al., 2002; Filippov et al., 2003). Migration of proliferating cells in SGZ occurs in a tangential form, parallel to the GCL, but also in a radial form, when newly-born cells migrate into the GCL. In the first days/weeks, only a few cells reach the outer third of the GCL, with the majority staying in the inner third of the GCL (Kempermann et al., 2003). The differentiation of the newly born cells occurs in several steps, which are identifiable with specific markers. The nomenclature of these steps changes, according to different classifications. It is possible to distinguish two main "categorizations": briefly, according to Gerd Kempermann there are four different stages of cell differentiation, type-

1, -2, -3 and calretinin stage cells; type-2 cells are further subdivided in -2a and -2b (Fig. 3) (Filipov et al. 2003; Kronenberg et al., 2003). In contrast, Alvarez-Buylla considers the following different stages of differentiation: B, D1, D2 and D3 cells (Fig. 3) (Seri et al., 2004). In both classifications, the differentiation stages are defined by the markers expressed by the cells, cell morphology, and its spatial position and orientation. The progenitor cells have astrocytic "properties" and are similar to radial glia cells (Seri et al., 2001), having triangular-shaped soma and a long and strong apical process that might reach the outer third of the granule cell layer (Fig. 3) (Filipov et al., 2003; Fukuda et al., 2003). Hippocampal radial glia-like cells are nestin- and glial fibrillary acidic protein- (GFAP) positive (Seri et al., 2001) being designated by type-1 or B cells, depending on the nomenclature (Fig. 3). Their daughter cells (type-2a cells) have a parallel orientation and are nestin positive (Fig. 3) (Filipov et al., 2003; Fukuda et al., 2003). Type-2b or D1 cells have a parallel orientation and are nestin and doublecortin (DCX) positive; these cells display already an early neuronal lineage, despite their progenitor identity (Fig. 3) (Kronenberg et al., 2003). Type-3 or D2 cells have all possible orientations between horizontal and vertical and are DCX but not nestin positive (Fig. 3) (Kronenberg et al., 2003). Cells in the latter stage are in a transition between a proliferation and a post-mitotic state. Cells that are in the calretinin or D3 stage have a round nucleus, are vertically orientated and are DCX, neuronal nuclei protein (NeuN) and calretinin positive (Fig. 3) (Brandt et al., 2003; Seri et al., 2004). Mature granule cells have a round nucleus, a vertical orientation and are NeuN and calbindin positive (Fig. 3) (Seri et al., 2004).

Functional integration implies the formation of dendrites and axons by the newly-born neurons to ensure the necessary connectivity with the pre-existing neuronal network. Axon elongation, and most of the dendritic tree formation occurs in the postmitotic phase of cell differentiation (calretinin or D3 stage and mature granule cells) (Hastings and Gould, 1999). Henriette van Praag and colleagues, in 2002, reported that newly-born neurons have electrophysiological properties similar to mature granule cells, which suggests that these cells are integrated in the system. The synaptic input that newly-born cells display changes as a result of the extension of their dendritic tree. GABAergic precedes glutamatergic input, which is consistent with the fact that GABAergic synapses have a somatic or proximo-dendritic location, while glutamatergic ones have a more distal distribution (Ambrogini et al., 2004).

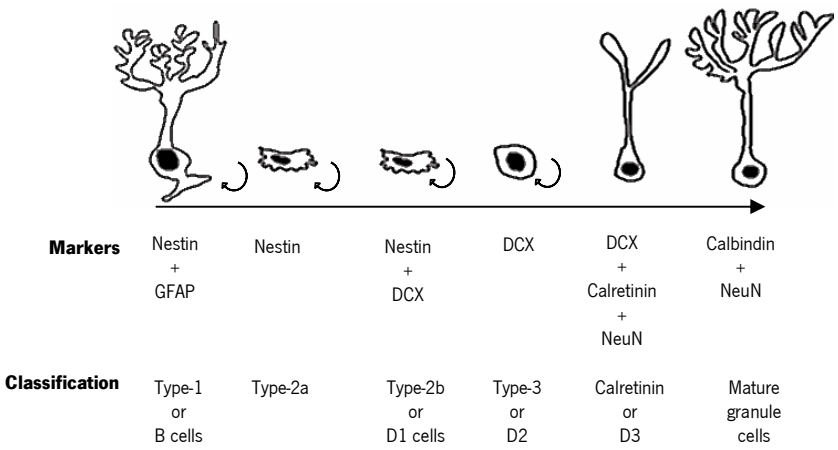

Fig. 3: Schematic representation of the proposed sequence of cell types in adult hippocampal neurogenesis according with the 2 existing classifications. Based on morphology, proliferative ability and expression of markers (nestin, GFAP, DCX, calretinin, calbindin and NeuN), six stages of neuronal development in the adult hippocampus can be identified based on Kemperman et al., (2004), or 5 stages according to Seri et al., (2004).

1.4 Modulation of adult neurogenesis

Organisms need to adapt to environmental changes in order to survive. The adaptation process requires a permanent interaction with the surrounding world, leading to behavioural adjustments that occur as result of physiological and/or anatomical changes. Being influenced by several factors (Table 1), adult neurogenesis represents another plastic possibility for the organism to adapt to its environmental contexts. In this thesis it will be emphasized the modulating actions of stress and corticosteroids, growth factors and lithium in adult hippocampal neurogenesis. The analysis is composed by an evaluation of each of these factors *per se* but also by their interactors.

1.4.1 Stress and corticosteroids

Response to stress is a homeostatic process that allows the organisms to survive to challenging environments. Although it is largely advantageous in an acute context, it may become harmful if the duration or intensity of the stressors overtakes the physiological capabilities of the organism. Acute and chronic stress induce distinct physiologic responses. Acute stress provokes an increase of catecholamines which raise frequency and debit of the heart (Landsberg and Youg,

1994) that favours the reorientation of blood flow to the brain, skeletal muscles and heart. Sustained stress stimulates the production of corticosteroids, leading to metabolic and immunologic changes (Sapolsky, 1992), which may induce secondary effects in the periphery and in the central nervous system (McEwen et al., 1997; Sousa et al., 1999; Schimmer and Parker, 2001).

Table 1. Factors influencing adult hippocampal neurogenesis.

Factor		**Effect**	**References**
Drugs	Lithium	+	Chen et al., 2000
Environmental enrichment		+	Kempermann et al., 1997
Growth factors	BDNF	+	Pencea et al., 2001
	EGF	+	Kuhn et al., 1997
	FGF-2	+	Kuhn et al., 1997
	IGF-1	+	Alberg et al., 2000
	TGF-β	+/-	(Anchan, et al., 1995; Wachs et al., 2006)
	VEGF	+	Jin et al., 2002
Hormones	Corticosteroids	-	Cameron et al., 1994
	Estrogens	+	Rasika et al., 1994
	Testosterone	+	Nordeen and Nordeen, 1989
Ischemia		-	Takagi et al., 1999
Neurotransmitters	Acetylcholine	+	Cooper-Kuhn et al., 2004
	Dopamine	+	Höglinger et al., 2004
	GABA	-	Jiang et al., 2003
	Glutamate (NMDA-receptors)	-	Gould et al., 1994
	Glutamate (KA-receptors)	+	Parent et al., 1997
	Norepinephrine	+	Kulkarni et al., 2002
	Serotonin	+	Brezun and Daszuta 1999
Physical activity	Running	+	Van Praag et al., 1999
Seizures		+	Bengzon et al., 1997
Stress	Chronic-mild-stress	-	Alonso et al., 2004
	Electrical foot shocks	-	Malberg and Duman, 2003
	Psychosocial	-	Gould et al., 1997
	Predator odour	-	Tanapat et al., 2001
	Restrain	-	Pham et al., 2003,
	Social isolation	-	Lu et al., 2003,

The main production center of corticosteroids are the adrenal glands, where both type of corticosteroids (mineralocorticoids and glucocorticoids) are synthesized from cholesterol. Contrary to aldosterone, the major mineralocorticoid, whose actions are largely restricted to kidneys (Funder et al., 1996), corticosterone (Cort) (cortisol in humans), the major glucocorticoid, (Williams and Dluhy, 1994) acts mainly in the energetic metabolism (Schimmer and Parker, 2001). Importantly, it should be remembered that Cort has also a mineralocorticoid profile (Williams and Dluhy, 1994), and that there are studies reporting the production of aldosterone in the brain (MacKenzie et al., 2002; Gomez-Sanchez et al., 2005).

Corticosteroids have two different receptors, mineralocorticoid (MR) and glucocorticoid receptor (GR). Aldosterone binds to MR while corticosterone binds to both receptors but with a higher affinity to MR (Reul and De Kloet, 1991). Hence, in basal conditions there is greater occupancy of MR than GR, while in a stress context both receptors reach a similar occupancy (Joels and De Kloet, 1994). After their activation MR and GR translocate from the cytoplasm to the nucleus (Truss and Beato, 1993; Lombes et al., 1994), where they form homo or heterodimers before binding to specific hormone response elements (HRE) of the DNA, to induce transcription alterations (Truss and Beato, 1993; Trapp et al., 1994). Besides modulating genomic function, corticosteroids are believed to have also plasmatic membrane receptors, which appear to influence the mitogen-activated protein kinase (MAP kinase) cascades (Makara and Haller 2001; Li et al., 2001).

Prolonged exposure to stress or corticosteroids, both in animals and in humans, have diverse deleterious effects (Starkman et al., 1992; Conrad et al., 1996; Keenan et al., 1996; Diamond et al., 1999; Sapolsky et al., 2000; Sousa et al., 2000; Waber et al., 2000), including impairment of some functions of the CNS. Neurological consequences of chronic stress or hypercortisolemia include decreased LTP (Pavlides et al., 1993), impoverishment of the dendritic trees (Sousa et al., 2000), increased apoptosis (Gould et al., 1991b) and decreased neurogenesis (Gould et al., 1991a). Although still controversial, some reports have suggested a role for post-natal neurogenesis in hippocampal-dependent memory (Gould et al., 1999b; Shors et al., 2001); indeed there is evidence that: i) learning enhances hippocampal neurogenesis (Gould et al., 1999a), ii) animals with increased neurogenesis have stronger LTP and better performance in memory tasks (van Praag et al., 1999), and iii) animals with decreased neurogenesis have a poor

memory performance (Lemaire et al., 2000; Shors et al. 2001). The mechanisms through which modulation of neurogenesis may influence learning and memory are, however, more enigmatic. Considering that the hippocampal formation is involved in learning and memory processes, it becomes obvious that hippocampal neurogenesis may contribute to the re-arrangement of hippocampal circuitry and allow a new level of learning (Kempermann et al., 2002; Chambers et al., 2004). It is, however, important to recall that neurogenesis occurs in parallel with an opposite event: cell death, mainly through apoptosis. Hence, the hippocampal balance between neurogenesis and apoptosis appears as an important factor to its functionality.

The hippocampus is also an important brain center involved in the negative-feedback control of the HPA axis; alterations of its functionality may, thus, deregulate this control (Sapolsky et al. 1986). Given that some drugs that normalize HPA axis (Gillespie et al., 2004) also increase neurogenesis (Malberg et al., 2000), this event may contribute to the hippocampal restructure and, consequently, to the restitution of the proper control of the HPA axis.

A final note to remember that neurogenesis and apoptosis are belived to share common regulatory factors (for review see Lucassen et al., 2006). However, the alterations in the rates of post-natal hippocampal neurogenesis and apoptosis induced by variations in the plasmatic concentration of corticosteroids may result from different pathways. Among these, it is possible to emphasize the changes in the levels of glucose/glycogen, pro and anti-apoptotic proteins and growth factors (for review see Sousa and Almeida 2002), as presented below.

1.4.1.1 Corticosteroids and glucose/glycogen regulation

One of the major actions of corticosteroids is their ability to modulate cellular glucose uptake (Sapolsky 1996). This seems to be supported by reports that indicate an increase in glucose consumption in ADX animals (Kadekaro et al., 1988), with an associated decrease in cerebral glucogenesis (Plaschke et al., 1996). Variations in glucose levels induce alterations in several hormones, namely insulin (Gilliagn et al., 1996), which is known to activate Akt and, consequently, inactivate the enzyme glycogen synthase kinase-3 (GSK-3) (Cross et al., 1995; Saltiel and Kahn, 2001). Indeed, changes in the levels of glucose were shown to induce a modulation of Akt and GSK-3 activity (Clodfelder-Miller et al., 2005). As explained in greater detail in subsequent sections (1.4.2.2), glycogen synthase kinase-3 has two isoforms (GSK-3α and

GSK-3β), and besides its effects on glycogen synthesis, GSK-3 has several other actions in intracellular signalling systems (for review see Grimes and Jope, 2001a). Interestingly, it seems that one possible mechanism of GSK-3β regulation is through GR activation (Sotiropoulos et al., 2006). In turn, there seems to be also feedback control in which GR is phosphorylated by GSK-3β, an alteration that inhibits the transcriptional activity of the receptor (Rogatsky et al., 1998).

GSK-3β appears also to be a central modulator of neurogenesis and apoptosis pathways through its actions in β–catenin and cyclic AMP response element binding protein (CREB) (Sakanata et al., 1998; Grimes and Jope, 2001b). GSK-3β represses β–catenin induction of neurogenesis by blocking the entry of this Wnt pathway protein in the nucleus, (Lie et al., 2005). CREB inhibition by GSK-3β influences not only neurogenesis, through modulation of brain derived neurotrophic factor (BDNF) production (Mai et al., 2002), but also increases apoptosis by the down regulation of the B-cell CLL/lymphoma 2) (Bcl-2) pathway (Wilson et al., 1996; Bonni et al., 1999).

1.4.1.2 Corticosteroids and pro and anti-apoptotic proteins

Hippocampal cell death apparently occurs due to exclusive GR activation. This hypothesis seems to be supported by observations in which neither stressful events nor high levels of exogenous Cort origin a noteworthy loss of neurons in the hippocampus (Sousa et al., 1998; Haynes et al., 2001), whereas Dex treatment induce a decrease in dentate gyrus' cell numbers (Sousa et al., 1999), as a result of increased apoptosis (Hassan et al., 1996; Almeida et al., 2000). In ADX animals, MR activation with aldosterone reverted granule cell death (Woolley et al., 1991) indicating a MR neuroprotective role; furthermore, MR activation also counterbalances the GR activation-associated cell death (Hassan et al., 1996; Sousa et al., 1999; Almeida et al., 2000). Hence, it seems that the final balance between MR *vs* GR occupation is a a relevant factor for hippocampal cell numbers (Sousa and Almeida, 2000).

The Dex-induced hippocampal apoptosis indicates that GR activation may lead to modifications in the expression of apoptotic pathway proteins. Almeida and colleagues (2000) showed that whereas Dex increased the ratio of the pro-apoptotic protein BCL-2-associated X protein (Bax) to the anti-apoptotic proteins Bcl_2 or Bcl-xL and the expression of p53, MR activation triggered the opposite effects. ADX-induced apoptosis also seems to be related to a raise in the p53 expression (Schreiber et al., 1994) and to Bax / Bcl-2 ratio (Conde et al., unpublished observations). In

addition, a recent finding by Zhou and colleagues (2005) revealed that B-cell-CLL/lymphoma2-associated athanonege (BAG-1) modulates the GR action, thus reinfoircing the interaction between Bcl_2 family proteins and corticosteroids.

1.4.1.3 Corticosteroids and growth factors

Growth factors are signalling molecules, involved in several neuronal cell events such as growth, division, axon sprouting (Maness et al 1994) and apoptosis (Sloviter et al., 1993). Hence, alterations in the levels of growth factors may induce changes in these events.

Although corticosteroids interact with several growth factor families, including: neurotrophins (nerve growth factor, NGF; brain derived growth factor, BDNF; neurotrophin 3, NT3 and neurotrophin 4/5, NT4/5), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (for review see Chao et al., 1994; MacLay et al., 1997; Machein et al., 1999), they do not influence similarly all growth factors. As examples, hypercortisolemia and ADX were reported to down-regulate NGF and BDNF (Siminoski et al., 1979; Barbany and Persson, 1993; Smith et al., 1995) but also to increase FGF (Hasson et al., 2000). Of notice for this work, VEGF levels were decreased with Dex (in vitro) (Machein et al., 1999) but did not change with ADX (Sibug et al., 2002). Considering that i) all of the above mentioned growth factors have been shown to protect from apoptosis (NGF (Sinson et al., 1997); BDNF (Almeida et al., 2005); bFGF (Jin et al., 2005); VEGF (Schanzer et al., 2004)), ii) that most increased neurogenesis (BDNF (Pencea et al., 2001); bFGF (Kuhn et al., 1997); VEGF (Jin et al., 2002)) and iii) taking into account the deleterious effects of corticosteroids in hippocampal plasticity, it is possible to infer that the corticosteroids-induced alterations in growth factor levels, may be one of the possible mediators of corticosteroids' action in hippocampal neuronal birth and death. A brief review of the influence of growth factors in cell birth, survival and death will follow.

1.4.2 Growth Factors

Growth factors are extracellular signal molecules that influence cell growth and survival. These factors were divided in different families (Barde, 1989) (Table 2), which generally bind to members of a restrict receptor group (Barbacid, 1994). Herein, their influences in neuronal

plasticity will be explored namely when related to adult neurogenesis; emphasis will be given to the role of vascular endothelial growth factor.

Table 2. Main growth factor families and their major elements and receptors (for review see Lewin and Barde, 1996, Cameron et al., 1998; Robinson and Stringer, 2001; Anderson et al., 2002; Wong and Guillaud, 2004).

Growth factors	Receptors
Neurotrophins family	
Neuronal Growth Factor (NGF)	trkA, p75
Brain Derived Growth Factor (BDNF)	trkB, p75
Neurotrophin-3 (NT-3)	trkC, p75
Neurotrophin-4/5 (NT-4/5)	trkB, p75
Neurotrophin-6 (NT-6)	trkA, P75
Fibroblast Growth Factor family	
Fibroblast Growth Factor-2 (FGF-2)	FGFR-1, -2, -3 and -4
Epidermal Growth Factor family	
Epidermal Growth Factor (EGF)	EGFR
Insulin Growth Factor family	
Insulin Growth Factor (IGF-1)	IGF-1R, IR
Transforming Growth Factor family	
Transforming Growth Factor-β (TGF-β)	Tβ1, Tβ2
Vascular Endothelial Growth Factor family	
Vascular Endothelia Growth Factor-A (VEGF-A)	VEGFR-1, VEGFR-2, NRP1, NRP2, HSP

1.4.2.1 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a key glycoprotein in angiogenenic process (generation of new blood vessels from pre-existing ones) (for review see Ferrara and Davis-Smyth, 1997). VEGF gene family includes five members (VEGF-A, -B, -C, -D and –E) that bind to six receptors (VEGFR-1, VEGFR-2, VEGFR-3, neuropilin-1, neuropilin-2 and heparan sulphate proteoglycan) (for review see Robinson and Stringer 2001). Recently, besides its role in angiogenesis, VEGF was reported to act also in the nervous system, namely as a protector factor against amyotrophic lateral sclerosis (Lambrechts et al., 2004).

The hippocampal formation structure and function is also likely to be influenced by VEGF, since VEGF-A and its receptors were described as being expressed by the several hippocampal cell populations (astrocytes, neurons and neuronal progenitor cells) (Meurer et al., 2003; Yang et al., 2003; Kutcher et al., 2004; Meng et al., 2006). In accordance, VEGF-A was reported to influence neuronal plasticity, by increasing neurogenesis both *in vitro* and *in vivo* (Palmer et al., 2000; Louissant et al., 2002; Jin et al., 2002) and by being an attractive guidance cue for the migration of undifferentiated neuronal cells (Zhang et al., 2003). Supporting the role of VEGF in neurogenesis, antidepressants (which are known to increase neurogenesis) were recently shown to raise VEGF levels (Warner-Schmidt and Duman, 2007); in contrast, stress and Dex (which decrease neurogenesis) were reported to reduce VEGF protein expression (Machein et la., 1999; Koedam et al., 2002; Heine et al., 2005; Wu et al., 2006). Interestingly, β-catenin was recently shown to influence the VEGF levels (Skurk et al., 2005). Therefore, it is plausible that VEGF expression can be modulated by lithium and stress, through regulation of GSK-3β. Effectively, both factors, stress (centrally) (Heine et al., 2005) and lithium (in the myocardium) (Kaga et al., 2006) increased VEGF expression. In the latter experimental condition, the raise of VEGF was paralleled by a decrease in GSK-3β and by an augment of β-catenin levels (Kaga, et al., 2006). Therefore, it seems of interest to analyse the effects of lithium in VEGF hippocampal levels both in basal and after stress exposure.

1.4.2.2 Other growth factors

Growth factors from other families, namely, fibroblast growth factor-2 (FGF-2), also called basic fibroblast growth factor (bFGF); insulin growth factor-1 (IGF-1); epidermal growth factor (EGF); transforming growth factor-β (TGF-β) and brain derived neurotrophic factor (BDNF), are also known to influence neuronal plasticity (Cameron et al., 1998; Pencea et al., 2001). The fibroblast growth factor family is composed by 23 members, which signal through 4 tyrosine kinase receptors (for review see Reus et al., 2003). FGF-2, which is expressed in the adult SVZ and hippocampal dentate gyrus (Ozawa et al, 1996), is a potent inducer of neurogenesis, both *in vivo* (Kuhn et al., 1997; Raballo et al., 2000; Yoshimura et al., 2001) and *in vitro* (Ghosh and Greenberg, 1995). IGF-1, whose production also occurs in the brain (Bondy et al., 1993), is a polypeptide hormone with a structure similar to insulin (Isaksson et al., 1991). Although IGF-1 also binds to insulin receptor (IR), its main receptor is the type 1 IGF receptor (IGF-1R) (Butler et al. 1998), which in the brain, is mainly located in the hippocampal dentate gyrus (Lesniak et al.,

1988). IGF-1 seems to be important for proliferation and neuronal maturation, during development (Carson et al., 1993; Beck et al., 1995), and in post-natal brain (Jian et al., 1998; Brooker et al., 2000; Aberg et al., 2000; Trejo et al., 2001). EGF has mitotic effects, but these change with cell populations, according to the expression of EGF receptor (EGFR), which is species-, region, and time-dependent (Palmer et al., 1995; Kirschenbaum et al., 1994; Represa et al., 2001). Although in adult rats EGFR is mainly expressed in the SVZ and SGZ (Seroogy et al., 1995) and a single EGF brain application had increased the number of SGZ proliferating cells (Tanapat and Gould 1997), chronic EGF infusion did not induce effects in SGZ proliferation (Kuhn et al., 1997). The TGF-β family signal through receptors with serine-threonine protein kinase activity (for review see Massagué et al., 1996). The TGF-β proteins are widely distributed in the brain (Unsicker et al., 1991) and their activity is contextual. Depending on the cell lineage and milieu, TGF-β proteins may induce proliferation (Anchan et al., 1995), differentiation (Battista et al., 2006) or even reduced neurogenesis (Zhang et al., 1997; Wachs et al., 2006; Buckwalter et al., 2006).

Neurotrophins comprise a family that share a homology superior to 50%, and include brain neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (NT-6). These factors bind to a high affinity group of tyrosine kinase-containing receptors (trkA, trkB and trkC) and to a lower-affinity transmembrane receptor (p75) (Chao et al., 1995). Although neurotrophins have similar actions they do not induce precisely the same effects. BDNF is known to increase adult neurogenesis (Pencea et al., 2001; D'Sa and Duman, 2002), synaptic transmission (Kang et al., 1995) and positively contribute to long-term potentiation (Korte et al., 1996). However, the other members of these family seem not to have such an essential role in potentiating adult neurogenesis (Frielingsdorf et al., 2007), but possess more stricking effects in neuronal survival (Lewin and Barde, 1996; Barnabe-Heider and Miller, 2003) and maintenance of the nervous system (Ghosh and Greenberg, 1995; Memberg and Hall, 1995; Chalazonitis, 1996; Li et al., 1997).

1.4.3 Lithium

Lithium (Li), a monovalent ion from the alkali metals, was first discovered by Johan August Arfvedson in 1817 during an analysis of petalite ore ($LiAl(Si_2O_5)_2$). Lithium as therapeutic tool was first used in 1840 when Lipowitz and Ure described its ability to treat bladder stones (El-Mallakh and Jefferson 1999). The use of lithium to treat psychiatric disorders was first reported in 1873 by William Hammond, referring the use of lithium bromide for mania. Lithium actions in depression were shown by Carl Lange in 1886, but it was only with John Cade's work (1949) that lithium arose as an effective therapeutic agent. Nowadays, lithium continues to be an effective treatment for mood disorders, namely bipolar disorder. Around 70 to 80 % of bipolar patients respond to lithium treatment alone. However, in maniac patients, benzodiazepines or antipsychotics are used simultaneously with lithium to trigger immediate maniac relief, as the therapeutic response to lithium requests 1 to 3 weeks (Kaplan and Sadock, 1998). Lithium has several side effects, including renal, thyroid, cardiac, dermatologic, and neurologic (cognitive and motor (tremors)) (Kaplan and Sadock, 1998). The occurrence of these effects is dependent on lithium plasmatic levels (above 2.5 mEq/L it becomes toxic). Physiologically, lithium is absorbed in the gastrointestinal tract, reaches its peak in 1 to 1.5 hours and stabilizes after 5 to 7 hours having a half-life of 20 hours (Kaplan and Sadock, 1998). Lithium is mainly excreted in the kidneys where is absorbed in the proximal tubes (Kaplan and Sadock, 1998).

Although it has been used in clinical practice for several decades, the mechanisms through which lithium induces its therapeutic actions are still not completely understood. Among the several pathways influenced by lithium, in this work only the interplay with phosphomonoesterases, glycogen synthase kinase-3 and corticosteroids will be explored.

1.4.3.1 Lithium and Phosphomonoesterases

The phosphoinositol (PI) signal pathway is involved in the modulation of several intracellular events and is regulated by the inositol polyphosphate 1-phosphatase (IPPase) and the inositol monophosphate phosphatase (IMPase); these enzymes control the synthesis and recycling of inositol, an essential element of this pathway (Leech et al. 1993). After activation of extracellular receptors coupled to G proteins that trigger the phospholipase C (PLC), this enzyme regulates the hydrolysis of phosphoinositide 4,5-biphosphate (PiP_2) to the second messengers diacylglycerol

(DAG) and inositol-1, 4, 5-triphosphate (IP_3) (for review see Majerus et al., 1992) (Fig. 4). DAG activates protein kinase C (PKC), while IP_3 causes the release of calcium from intracellular stores. The recycling of IP_3 to inositol, a precursor of PiP_2 is essential in cells where inositol is not freely available. Both IPPase and IMPase catalyse critical steps in the inositol recycling, maintaining the IP pathway available (Fig. 4).

The "inositol depletion hypothesis" for lithium actions arose due to the low rate of inositol crossing the blood brain barrier and/or the cell membrane of some neuronal populations (Berridge et al., 1989; Gain et al., 1993). This hypothesis suggests that by inhibiting IPPase and IMPase, through competition with magnesium (at a Ki of 0.8 mM) (York et al., 1995), lithium decreases the levels of PiP_2 usable for signalling (Berridge et al., 1989) and consequently would have mood stabilizing effects.

Despite the reports suggesting that depletion would not play an important effect on PI-mediating signal (Berry et al. 2004), lithium effectively reduces free inositol concentration in the brain of rodents and human and, thus, the "inositol depletion hypothesis" is still a possible explanation for the mood stabilizer effects of lithium (Quiroz et al., 2004).

1.4.3.2 Lithium and Glycogen synthase kinase-3

Glycogen synthase kinase 3 (GSK-3) is a serine/therionine protein kinase that acts in the regulation of glucose metabolism through the phosphorylation of glycogen synthase, inhibiting its activity (Roach, 1990). GSK-3 is also involved in several biological processes, including early embryo development, oncogenesis, cell death. It is also implicated in CNS disorders, namely in psychiatric disorders (as bipolar mood disorder) and in neurodegenerative diseases (as Alzheimer's disease) (for review see Grimes and Jope, 2001). Hence, GSK-3 has become a novel target for old therapeutic needs (Eldar-Frinkelman, 2002; Bhat et al., 2004; Gould et al., 2004).

1.4.3.2.1 Substrates

GSK-3 has two isoforms (GSK-3α and GSK-3β), which share 98% homology in their catalytic domain (Woodgett, 1990). GSK-3β expression occurs in all tissues, including in the brain (Woodgett, 1990), where it regulates several proteins (Table 3). From these, due their relevance to neurogenesis and/or apoptosis processes, four will be highlighted: β-catenin, cyclic AMP

response element binding protein (CREB), glucocorticoid receptor (GR), and heat shock factor-1 (HSF-1) (Fig. 4).

i) β-Catenin is a cell adhesion molecule that fixes cadherins during the formation of cell junctions (Ozawa et al., 1989). However, this protein also displays a significant role in cell signalling, namely in the Wnt pathway. Without the activation of Wnt signalling, β-catenin is found in a cytoplasmatic complex with adenomatous polyposis coli protein (APC), axin, protein phosphatase 2A (PP2A) and GSK-3β (Kikuchi, 1999). GSK-3β was shown to regulate β-Catenin; through phosphorylation, it decreases the levels of β-catenin (Yost et al., 1996), as it directs β-catenin for ubiquitination and degradation via the proteosome pathway (Fig. 4). The stabilization and accumulation of β-catenin, through dishevelled (dsh) action, as a result of activation of the Wnt pathway, induces the migration of β-catenin to the nucleus where it forms a transcriptional complex with members of the T-cell factor (TCF) / lymphoid enhancer factor (LEF) family (Miller et al., 1999). This complex binds to DNA and regulates the expression of several genes. Lithium, due to its ability to inhibit GSK-3β, induces similar results in β-catenin levels to those of the Wnt pathway activation (Klein and Melton, 1996).

ii) CREB is involved in several cellular events (e.g. long-term memory, synaptic plasticity, apoptosis) (Davis et al., 1996). It also promotes cell survival, by increasing the expression of the anti-apoptotic protein bcl-2 (Wilson et al., 1996). Phosphorylation of CREB at Ser-133 is essential for its activation (Gonzalez and Montminy, 1989). However, phosphorylation of CREB at Ser-133 also creates a consensus site at Ser-129 that permits GSK-3β phosphorylation (Wang et al., 1994). Hence, GSK-3β seems to de-activate CREB, instead of blocking its activation. Increased levels of GSK-3β were reported to decrease the activity of CREB (Grimes and Jope 2001); the inhibition of CREB activation, in turn, was being shown to increase apoptosis (Bonni et al., 1999). On the other hand, lithium, which inhibits GSK-3β, raised CREB DNA binding activity *in vitro* an in several regions of rat brain (Ozaki and Chuang, 1997). In summary, CREB DNA binding activity seems to have a complex regulation, which includes an inhibitory action of GSK-3β that can be influenced by lithium (Fig. 4).

Table 3: Mammalian proteins phosphorylated by GSK-3β (Adapted from Grimes and Jope 2001).

Signalling Proteins	Structural Proteins	Transcription factors
Acetyl-CoA carboxylase (Hughes et al.,1992)	Dynamin-like protein (Hong et al., 1998)	AP-1 (Jun family) (Boyle et al., 1991)
Amyloid precursor protein (Alpin et al., 1996)	Microtubule associated protein 1B (MAP1B) (Garcia-Perez et al., 1998)	β-catenin (Rubinfiled et al., 1996)
APC (Rubinfeld et al., 1996)	Microtubule associated protein 2 (MAP2) (Garcia-Perez et al., 1998)	CCAAT/enhancer binding protein (C/EBPα) (Ross et al., 1999)
ATP-citrate lyase (Hemmings et al., 1982b)	Myelin basic protein (Yu and Yang, 1994)	Cyclic AMP response element binding protein (CREB) (Wang et al,. 1994c)
Axin (Hart et al., 1998)	Neuronal cell-adhesion protein (NCAM) (Mackie et al., 1989)	Glucocorticoid receptor (rat) (Rogatsky et al., 1998)
Cyclic AMP-dependent protein kinase (Hemmings et al., 1982a)	Neurofilaments (Guan et al., 1991)	Heat shock factor 1 (HSF-1) (Chu et al., 1996)
Cyclin D1 (Diehl at al., 1998)	Ninein (Hong et al., 2000)	Myc (Saksela et al., 1992)
Eukaryotic initiation factor 2B (eIF2B) (Welsh and Proud, 1993)	Tau (Hanger et al., 1992)	NFkB (Hoeflich et al., 2000)
Glycogen synthase (Embi et al., 1980)	Synapsin (Hall et al., 2002)	Nuclear factor of activated T cells (NFAT) (Beals et al., 1997)
Insulin receptor substrate-1 (IRS-1) (Eldar-Finkelman and Krebs, 1997)		
Myelin basic protein (Yang et al., 1986)		
NGF receptor (Taniuchi et al., 1986)		
Protein phosphatase 1 (Fiol et al., 1988)		
Protein phosphatase inhibitor-2 (Hemmings et al., 1982b)		
Pyruvate dehydrogenase (Hoshi et al., 1996)		

iii) Glucocorticoid receptor (GR) activation may induce or repress transcriptional initiation (Miner et al., 1990). GR activity seems to be regulated through phosphorylation (Bodwell et al., 1995) in four residues (Krstic et al., 1995) by kinases of the serine/threonine-proline-directed family, in which GSK-3 is included. GSK-3, but not cyclin-dependent kinases (Cdk), nor c-Jun N-terminal kinases, phosphorylates rat GR's threonine 171 (Thr^{171}) (Rogatsky et al., 1998). This phosphorylation was observed to be dose dependent, and to inhibit GR-induced transcriptional enhancement, but not repression (Rogatsky et al., 1998). GSK-3-dependent inhibition of GR-mediated increase transcriptional activity can reach up to 50% of controls (Rogatsky et al., 1998). Considering that Akt, which is a GSK-3 inhibitor, was shown to raise GR transcriptional enhancement (Rogatsky et al., 1998), lithium, which also inhibits GSK-3, may also influence GR activity. Therefore, lithium's neuroprotection and plasticity could occur also through modulation of GR functions, as supported by the reported lithium-induced increase of GR mRNA (Semba et al., 2000).

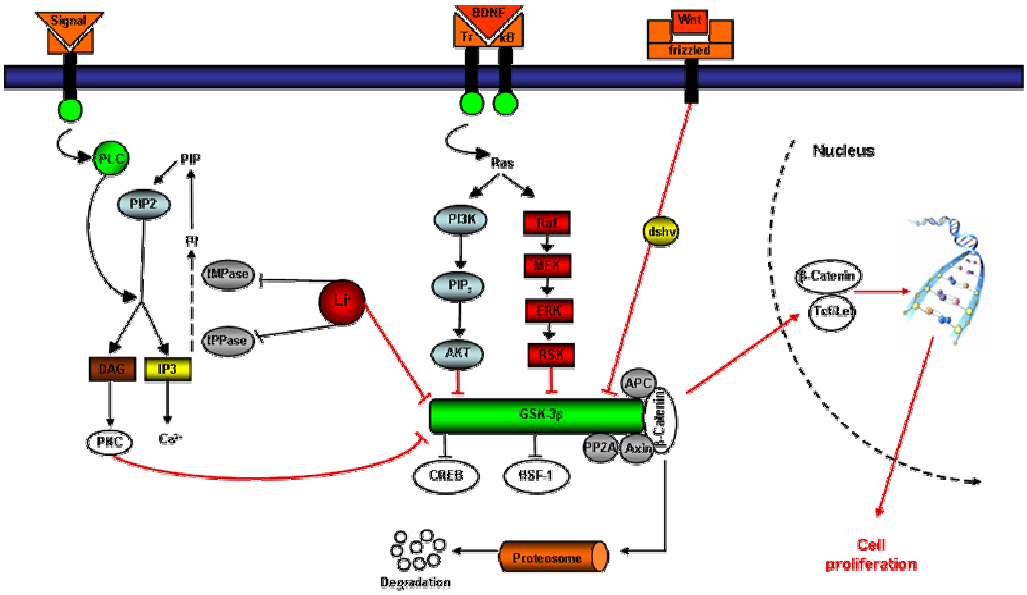

Fig. 4: Schematic representations of lithium interactions with the phosphoinositol (PI) signal pathway and glycogen synthase kinase-3β. It is also possible to observe the molecular effects of GSK-3β normal action (black arrows) and when inhibited (red arrows).

iv) The transcription factor heat shock factor-1 (HSF-1) was reported to modulate cellular defence mechanisms as response to lethal insults (e.g. oxidative stress, heavy metals, high temperatures, etc). The activation of HSF-1 controls heat shock proteins' expression (e.g. hsp70), which preclude protein intercellular accumulation and aggregation due to its capacity to chaperone misfolded proteins (for review see Grimes and Jope, 2001). The regulation of HSF-1 activity is controlled by modifications induced through phosphorylation and dephosphorylation, including its inhibition by GSK-3β due to the phosphorylation of HSF-1' Ser-303 (Fig. 4) (Chu et al., 1996). Through the inhibition of GSK-3β, lithium increases the activation of neuronal HSF-1 and the levels of heat shock proteins (Bijur and Jope, 2000). This effect is even more relevant when considering Zhou and colleagues (2005) work, where they report a lithium-induced increase of BAG-1 [Bcl-2 (B-cell CLL/lymphoma 2)-associated athanogene] levels. BAG-1 is a protein from the anti-apoptotic Bcl-2 family, which potentiates Bcl-2 anti-apoptotic actions (Takayama et al., 1995), cochaperones GR (Schneikert et al., 1999) and interacts with hsp-70 (Takayama et al., 1998).

In conclusion, the influence of lithium upon GSK-3β actions in proteins involved in neurogenesis and apoptosis pathways, supports the hypothesis that modulation of GSK-3β is one possible mechanism through which lithium promotes neuroplasticity.

1.4.3.2.2 Regulation

As many other enzymes, GSK-3 regulation occurs through several mechanisms, including, protein complex formation, intracellular localization and phosphorylation. GSK-3 inhibition is dependent on phosphorylation of a serine residue (Ser9 in GSK-3β and Ser21 in GSK-3α) located in the N-terminal domain, while activation depends on tyrosine (Tyr-216) phosphorylation (Huges et al., 1993). There are some factors known to inhibit GSK–3β, which are activated through several signals (Table 4) (for review see Grimes and Jope, 2001). Lithium is known to inhibit GSK–3β (Stambolic et al., 1996), directly via competition with magnesium (Ryves et al., 2001), but it also induces a GSK–3β indirect inhibition through its actions in the IP pathway. Due to its inhibition of IMPase and IPPase activity there is an increase of PiP2 and, consequently, of DAG and PKC (Fig. 4), which inhibit GSK–3β (Goode et al., 1992) (Table 4). The effectiveness of chronic lithium treatment is possible to be observed through the increase in the levels of the GSK–3β down-stream target, β-catenin (Gould et al., 2004).

Table 4: GSK-3β inhibitory signals and effectors.

Signal	**Effectors (phosphorilators)**
BDNF (Bhave et al., 1999)	Akt (Shaw et al. 1997)
Endothelin-1 (Haq et al., 2000)	Akt (Shaw et al. 1997)
Epidermal growth factor (EGF) (Saito et al., 1994)	p90RsK; Akt (Suntherland et al., 1993; Shaw et al. 1997)
Insulin (Cross et al., 1995)	Akt (Shaw et al. 1997)
Insulin growth factor-1 (IGF-1) (Cui et al., 1998)	Akt (Shaw et al. 1997)
Lithium (Stambolic et al., 1996)	Lithium (Stambolic et al., 1996)
NGF (Pap and Cooper, 1998)	Akt (Shaw et al. 1997)
Oxidative stress (Blair et al. 1999)	Akt (Shaw et al. 1997)
Wnt (Cook et al., 1996)	PKC (Goode et al., 1992)
?	p70 S6 Kinase (Suntherland et al., 1993)
?	Protein Kinase A (Fang et al., 2000)

As previously said, lithium is a mood stabilizer used in clinical practice for several decades. Besides being inhibited by lithium, another evidence for a GSK–3β role in bipolar disorder, came from the report that valproic acid (another mood stabilizer) also inhibits GSK–3β, at therapeutic concentrations (Yuan et al., 2001). However, it must be noted that inhibition of GSK-3β by lithium, as by valproate, only reaches a 50% value (Klein and Melton, 1996), which suggests that therapeutic levels of lithium do not totally inhibit GSK–3β mechanisms and, consequently, do not completely arrest the essential cellular events induced by this enzyme.

One intriguing question about lithium refers to whether its neuroprotective actions contribute to its mood stabilizing effects. Recently, reports suggest that antidepressants increased adult hippocampal neurogenesis (Malberg et al., 2000). The lithium-induced modulation in the cellular turnover of hippocampal neurons is due not only to an increase in neurogenesis but also to a reduction in apoptosis (to which may contribute lithium-induced decrease of p53 (Chen and Chuang, 1999), and raise of Bcl-2 levels (Chen et al., 1999)). The precise mechanisms by which GSK–3β facilitates neuronal death remains unclear. However, the effects that GSK–3β exerts in several key proteins, including being an upstream protein of the pro-apoptotic protease caspase-3 and contributing to p53 activation (for review see Grimes and Jope, 2001), undoubtedly influences its pro-apoptotic actions. Concordantly, GSK–3β inhibition, induced by lithium (Bijur et al., 2000), was reported to attenuate or prevent apoptosis (Watcharasit et al., 2003) and to have antidepressant effects (Gould et al., 2004). Considering that, besides being used in the treatment of bipolar disorder, lithium is also employed in some cases of depression (Kaplan and Sadock, 1997), and taking into account its pro-neurogenesis and anti-apoptotic ability, it is plausible to consider that lithium "neuroprotective cellular actions" may be associated to its clinical effectiveness.

1.4.3.3 Lithium and Corticosteroids

The relationship between hypercortisolemia statuses and depression is well established as: (i) some depressive patients present high levels of cortisol (Gibbons et al 1962), (ii) some patients with Cushing´s syndrome present depressive symptoms (Sonino et al., 2001), (iii) around 50% of depressed patients have a deregulation of the HPA axis (assessed in the dexamethasone-supression test) (Kaplan and Sadock, 1987), (iv) GR antagonists display antidepressant effects (DeBattista and Belanoff, 2006), (v) hypercortisolemia induces a reduction in the concentration of brain's serotonin (for review see van Praag 2004), (vi) distinct classes of antidepressants (serotonin specific reuptake inhibitors, tricyclics and monoamine oxidase inhibitors) regulate the HPA axis (Reul et al., 1993 and 1994; Jensen et al., 1999) and (vii) neurogenesis, which is largely influenced by corticosteroids has been implicated in depression (Malberg et al., 2000). Therefore, it is possible to infer that hypercortisolemia and/or its associated mechanisms have a role in the etiology or maintenance of depression. In certain cases of major depression (basically in those refratory to treatment), lithium is used has an adjuvant to antidepressant therapy (Kaplan and Sadock, 1997). Lithium has also been shown to increase the expression of the

hippocampal GR mRNA (Semba et al., 2000), which may raise the sensitivity of the negative feedback control of the HPA axis and, thus, helping in its regulation. On the other hand, the activity of one of lithium's main targets (GSK-3β) was reported to be increased by GR (Smith et al., 2002), implying another possible mechanism for lithium action in the regulation of corticosteroids cellular effects. GSK-3β was also recently shown to reduce the levels of the pre-synaptic protein synapsin-I, an effect that was prevented by lithium (Hal et al., 2002). This decrease might contribute to the impaired synaptic transmition associated with the deleterious effects in the dendritic tree, including synaptic loss, induced by corticosteroids (for review see Sousa and Almeida 2002). Finally, the modulation of hippocampal neuronal plasticity by corticosteroids is known to be related with memory and learning deficits, which are symptoms commonly described by depressive patients (Marvel and Paradiso, 2004). Taken together, these different lines of evidence contribute to the understanding of the physiological importance of hippocampal neuroplasticity modulation by lithium and will be further explored in this thesis.

1.5 Aims

The main objective of this thesis was to study how can postnatal neurogenesis be modulated and its implications in health and in disease. In order to achieve this goal, several operational tasks were performed:

A) Establish, in basal conditions, the topography of neurogenesis and apoptosis in the hippocampal dentate gyrus of pre-pubertal and adult rats (Chapter 2.1);
B) Characterize the effects and some molecular pathways through which chronic mild stress (CMS) impacts hippocampal cell turnover, and its possible association to depressive-like behaviour (Chapter 2.2);
C) Determine the role of MR and GR in hippocampal cell fate and depressive-like behaviour (Chapter 2.3);
D) Explore the effects of CMS on VEGF-A expression (Chapter 2.4);
E) Ascertain lithium's ability to prevent CMS and corticosteroids milieu inbalances effects in hippocampal cell turnover by studying the modulation of the molecular pathways involved in hippocampal cell-fate and synaptic plasticity (Chapters 2.2, 2.3 and 2.4).

Chapter 2

Experimental Work

Chapter 2.1

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Mapping cellular gains and losses in the postnatal dentate gyrus:

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Mapping cellular gains and losses in the postnatal dentate gyrus: implications for neuropsychiatric disorders

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Abstract

Neurogenesis and apoptosis occur contemporaneously in the postnatal hippocampal dentate gyrus and have been implicated in mood and cognitive disorders; in particular, the former correlates with the manifestation of antidepressant effects, but its quantitative and topographical relationship with concomitant cell death has not been investigated. Accordingly, we applied stereological measurements to obtain synchronized topographical maps of these two events in rats aged 1 and 3 months under basal conditions; the two ages were chosen to represent neuro-developmental windows during which cell proliferation and death are occurring at peak and relatively steady levels, respectively. Our analysis shows that apoptotic cells are evenly distributed throughout the dentate gyrus, although the incidence of apoptosis decreased gradient-wise from the tip of the suprapyramidal layer and was highest in the external third of the granule cell layer; importantly, apoptosis was higher in the left hippocampus. In addition, we confirm previous less-stringent studies demonstrating that neurogenesis occurs differentially in the dorsal-ventral axis of the hippocampus and in suprapyramidal-infrapyramidal blades of the dentate gyrus. These results raise intriguing new questions regarding the coordinated regulation of hippocampal neurogenesis and apoptosis since the two processes apparently share common regulatory factors. In addition, these findings open questions with respect to the functional significance of topographical gradients in neurogenesis and apoptosis in the context of the etiopathogenesis of neuropsychiatric diseases and the reported dependence on the efficacy of therapeutic agents on the generation of new hippocampal neurons.

Key-words:

Hippocampal formation; plasticity; hemispheric asymmetry; apoptosis; proliferation; laterality

Introduction

The hippocampal formation, a component of the limbic system, is endowed with remarkable plasticity. In contrast to other brain regions, a significant number of neurons are generated after birth from a local population of progenitor cells in the dentate gyrus of the hippocampus (Altman and Das, 1965; Cameron et al., 1993; Kuhn et al., 1996). Simultaneously, the hippocampus displays physiological elimination of neurons, through apoptotic mechanisms. Thus, the cytoarchitecture of the dentate gyrus results from the balance between neurogenesis and the concurrent elimination of neurons through apoptosis (Gould et al., 1991; Lossi and Merighi, 2003); interestingly, these opposite processes share a number of common regulatory factors, e.g. adrenal steroids, excitatory amino acids and growth factors (Gould and McEwen, 1993; Cameron and Gould, 1994; Lu et al., 2003; Lu et al., 2005).

Neurogenesis is currently viewed as an important phenomenon in psychiatric disorders which are characterized by either degenerative changes, inappropriate neural development, or impaired neural plasticity, e.g. dementia, drug addiction, and schizophrenic and affective psychoses. Experiments showing that psychotropic drugs, including antidepressants and atypical antipsychotics, can significantly influence neurogenesis support this view. In the case of major depression, one current view is that the therapeutic efficacy of antidepressants may be contingent upon the generation of new neurons (Santareli et al., 2003), although numerous questions have been raised about such an interpretation (Malberg et al., 2000; Sapolsky, 2004).

Although neuronal birth in the postnatal dentate gyrus has attracted wide attention, there remains a conspicuous lack of information regarding the topographic distribution of apoptotic cells during the normal postnatal development of the dentate gyrus. Moreover, no studies have addressed the relationship between apoptosis and neurogenesis, two processes that overlap in their occurrence but also in features of their regulation. By creating a systematic topographic map of the incidence of apoptosis and neurogenesis in the hippocampus, one could expect to gain insights into the mechanisms underlying the functions attributed to cell acquisition and/or elimination of cells in the hippocampal formation. This view is illustrated by the demonstration of profound differences in the electrophysiological properties (Wang et al., 2000) and functions (Moser and Moser, 1998; Scharfman et al., 2002) of granule cells located in different regions of the hippocampus, as well the reported hemispherical differences in the volumes of the hippocampus in rodents (Verstynen

et al., 2001) and humans (Zaidel et al., 1998; Utsunomiya et al., 1999). Lastly, an analysis of the topographic distribution of cell birth and death can be expected to contribute to how these phenomena together contribute to the final cytoarchitectonic organization and function of the hippocampus.

The work described in this paper addresses the issue of whether cell proliferation and apoptosis in the dentate gyrus show subregion-specific patterns or, alternatively, occur in a stochastic fashion; we examined this in rats displaying different (1 and 3 month old animals) levels of both phenomena in order to avoid confounds from the a priori assumption that neurogenesis and apoptosis are closely-related events. Proliferation was assessed immunohistochemically after incorporation of the thymidine analogue bromodeoxyuridine (BrdU) into the DNA of S-phase cells and through the expression of histone 3 phosphorylated at serine 10 (pHisH3-Ser10); site-specific phosphorylation of histone H3 at serine 10 (Ser10) occurs exclusively and rapidly after mitosis (early G2 through to M-phase) in mammalian cells and the Ser10 remains unphosphorylated throughout interphase (Hendzel et al. 1997). We double-stained cells for GFAP or NeuN in order to determine the phenotypic fate (astrocytic vs. neuronal) of the newly-acquired cells. Apoptosis was evaluated using established morphological criteria coupled with terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labeling (TUNEL) histochemistry.

Materials and methods

Animals

Twenty male Wistar rats (Charles River, Barcelona, Spain), twelve aged 4 weeks (mean weight of 120g), and 8 aged 12 weeks (mean weight of 350g), were used in this study. Animals were housed under standard laboratory conditions (12 hours light cycle; 22°C, 55% humidity; food and water available ad libitum). Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Tissue preparation

In order to detect proliferating cells, six rats aged 1 month and four rats aged 3 months received daily intraperitoneal injections of 50 mg/kg BrdU (Sigma, St. Louis, MO) on 3 consecutive days. Animals were killed by rapid decapitation 24 hours after the last BrdU injection. All brains were carefully removed, placed in cryoprotectant and snap-frozen in liquid nitrogen. Serial coronal sections (20 μm), extending over the entire length of the hippocampus, were obtained using a cryostat and mounted on poly-L-lysine-coated slides. Another similar set of animals was used for TUNEL staining.

Histochemical procedures

Detection of apoptosis

To visualize apoptotic cells sections from six 1-month old and four 3-month animals were examined by TUNEL histochemistry (Fig. 1). All sections containing the hippocampal formation were stained. Sections were fixed as described above, permeabilized in a two-step procedure (0.1% trypsin in PBS, pH 7.2 at 37 °C, followed by 0.1% Triton X-100 in PBS, 5 min. at room temperature) and treated with 3% H2O2 in PBS (3 minutes) to block endogenous peroxidases. Sections were then pre-incubated in terminal deoxynucleotidyl transferase (TdT) buffer before incubation (37° C, 1 h) in a cocktail consisting of 13.5 μL TdT (MBI Fermentas, Hanover/MD), 6.75 uL dUTP-Biotin (Roche, Basel/Switzerland), 90 μL TdT buffer, 20 μL TdT enzyme buffer (MBI Fermentas) and 770 μL distilled water. Development and visualization was achieved using a commercial avidin-biotin/DAB system (Vector Labs, Burlinghame/CA). Hematoxylin was used as a counterstain.

Detection of BrdU and pHisH3-Ser10

BrdU incorporation was detected by immunocytochemistry on every 8th section containing the hippocampal formation (Fig. 2A). Briefly, sections were fixed in 4% paraformaldehyde (PFA) for 30 minutes, permeabilized for 10 minutes in a solution containing 0.2% Triton X-100 in Tris buffer saline (TBS), microwaved (20 minutes) while immersed in citrate buffer (0.1 M), and acidified in 2 M HCl (30 minutes). Endogenous peroxidase activity was blocked with 3% H2O2 in TBS (10 minutes) and non-specific staining was blocked with 4% bovine serum albumin (BSA) in TBS (30 minutes). Subsequently, sections were incubated overnight with a mouse monoclonal anti-BrdU (1:50, Dako, Glostrup/DK). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and diaminobenzidine (DAB: 0.025% and 0.5% H2O2 in Tris-HCl 0.05M, pH 7.2). Specimens were lightly counterstained with hematoxylin.

Immunocytochemistry for pHisH3-Ser10 was performed on every 8th section containing the hippocampal formation (Fig. 2B). Sections were fixed in 4% PFA, permeabilized with 0.3% Triton-X100/PBS, blocked in 3% donkey serum/0.3% Triton for 30 minutes and then incubated (4°C overnight) with primary antibody (1:100, Cell signaling, Beverly, MA). After washing, slices were incubated for 1 hour at room temperature with biotinylated anti-rabbit secondary antibody (1:500, Vector Labs, Burlinghame, CA). Sections were then washed and the reaction was developed and visualized with a commercial avidin-biotin/DAB system (Vector Labs, Burlinghame, CA).

Double staining forBrdU and differentiation markers

One set of a 1-in-16 series of hippocampal sections from each 1 month old animal was sequentially double stained for BrdU and NeuN (Fig. 3A) while another set was sequentially stained for BrdU and GFAP (Fig. 3B), using the above-mentioned protocol for BrdU labeling. For BrdU and NeuN double immunofluorescence, sections were incubated overnight with a rat anti-BrdU monoclonal antibody (1:50, Abcam, Cambrige/UK). After rinses in TBS, secondary antibody anti-rat Alexa Fluor-488 (1:200, Molecular Probes, Eugene/OR) was applied to the sections (2 h). Sections were then washed in TBS and incubated with mouse anti-NeuN (1:100, Chemicon, Temecula/CA) primary antibody (3 h). After washing in TBS, NeuN fluorescence visualization was accomplished by incubation for 2 h with anti-mouse Alexa Fluor-568 (1:200, Molecular Probes, Eugene/OR).

For the BrdU and GFAP double immunofluorescence labeling, sections were incubated overnight with mouse anti-BrdU monoclonal antibody (1:50, Dako, Glostrup/DK). After washing, sections were incubated with anti-mouse Alexa Fluor-568 (1:200, Molecular Probes, Eugene/OR) secondary antibody (2 h), washed in TBS and incubated with mouse anti-GFAP (1:200, Dako, Glostrup/DK) primary antibody (3 h). After rinsing in TBS, GFAP fluorescence visualized by incubation (2 h) with anti-rabbit Alexa Fluor-488 (1:200, Molecular Probes, Eugene/OR).

For each animal, 50-100 BrdU-positive cells within the dentate gyrus were analyzed after double-staining for BrdU and the respective neuronal or glial markers, using a Leica FT2 AOBS confocal microscope.

Stereological procedures

Estimation of cell density in the different subdivisions of the dentate gyrus (Fig. 4) was obtained using the StereoInvestigator software (MicroBrightField, Williston/VT). Every section stained (i.e. every 8th section for proliferating cells, all sections for apoptosis) was used for the estimations in the different areas of interest. Cells were counted using a x100 lens which provided sufficient resolution to easily identify the different layers and cells types. Cellular discrimination was based on morphological and staining criteria: apoptotic cells were identified by TUNEL coloration and, in addition, by their small size, chromatin condensation, and vesicular formation; cells that were positively labeled with BrdU or pHisH3-Ser10 were considered as cells which had been recently generated, i.e. within the timeframe of the experiment.

The following criteria were used to define transversal divisions within the granular cell layer (GCL) and subgranular zone (SGZ): the angular subdivision (Ang) was considered as the area extending from the tip of the crest up to the end of its curve; the remaining area was divided into two equal parts, delineating the intermediate (Med) and extreme (Ext) subdivisions (Fig. 4). These subdivisions were further distinguished in terms of their position within the suprapyramidal (Supra) and infrapyramidal (Infra) blades. Longitudinal divisions of the GCL (internal, intermediate and external) were defined by dividing the thickness of the GCL into 3 equal parts. The SGZ was considered to be a 3-cell-body-wide zone at the border between the GCL and hilus (HI). The division between dorsal and ventral hippocampus was based on the relative position of the dentate gyrus with respect to the third ventricle or cerebral aqueduct: areas located superior to a transverse plane passing at the top of these structures were considered dorsal, whereas those located inferior were considered ventral.

Statistics

The overall effect of topography on proliferating/apoptotic cells within the dentate gyrus was assessed using one- or two-way analyses of variance (ANOVA), followed by Tukey Post-hoc multiple comparisons test. Correlations between BrdU- and pHisH3-Ser10-positive cells were determined by Pearson's correlation test. The results are expressed as group means + standard error (SEM). SEM and coefficient of error (CE) were calculated accordingly to Gundersen and Jensen (1987). Statistical significance was accepted when p was ≤ 0.05.

Results

Volumes of the dentate gyrus

Analysis of the volumes of the different layers of the dentate gyrus of 1 month old animals revealed a slight non-significant predominance of the left dentate gyrus (Table 1), that was equalized/inverted in 3-month old rats. Comparisons between dorsal and ventral divisions of 1-month-old rats ($F(7,47) = 32.1$; $p \leq 0.0005$) showed increased volumes in all dorsal components of the hippocampal formation, except in the HI where the volume of the ventral division outweighed that of the dorsal division. No volumetric differences were found between dorsal and ventral divisions in 3-month old rats. Comparison of animals aged 1 or 3 months revealed increased volumes in every subdivision of the dentate gyrus except the SGZ, but only the molecular layer (ML) showed a significant change (Table 1).

Apoptosis

As illustrated in Fig. 1 and 5A and confirmed by ANOVA there were significant differences in the occurrence of apoptosis in the GCL and SGZ of the left and right hemispheres ($F(7,47) = 27.9$; $p \leq 0.0005$); the left dentate gyrus displayed higher indexes of apoptosis (Fig. 1, 5A, 5B).

In addition, comparison of densities of apoptotic cells between the main divisions of the dentate gyrus showed the SGZ to have the highest levels of apoptosis at both ages (1 month: $F(3,23) = 34.7$, $p \leq 0.0005$; 3 months: $F(3,15) = 90.0$; $p \leq 0.0005$). Age-comparisons proved that the rate of apoptosis in the GCL, SGZ and HI is significantly increased in older animals (GCL: $F(1,9) = 34.0$, $p \leq 0.0005$; SGZ: $F(1,9) = 39.4$, $p = 0.0005$; HI: $F(1,9) = 5.2$, $p \leq 0.05$) (Fig. 5C).

Apoptotic events also varied along the rostro-caudal axis of the dentate gyrus, but differences were only statistically significant in the SGZ of 1-month old rats ($F(1,11) = 11.3$; $p = 0.007$) (Fig. 1, 6). The degree of apoptosis in the supra- and infra-pyramidal blades of the dentate gyrus varied at both ages (1 month: $F(3,23) = 18.6$, $p \leq 0.0005$; 3 months: $F(3,15) = 21.9$, $p = 0.001$); apoptosis was higher in the supra-pyramidal blade of the GCL of 1-month old rats, but at 3 months of age, apoptosis was predominantly found in the supra-pyramidal blade of the SGZ (Fig. 6B), i.e. the spatio-temporal profiles of apoptosis was altered with increasing age.

By subdividing the supra- and infra-pyramidal blades further, we found the highest index of apoptosis to occur in the extreme portion of the supra-pyramidal blade (Fig. 5D, 6B).

Further, comparison of animals aged 1 and 3 months showed that higher levels of apoptosis occur in the Sup Intmed, Sup Ang, Inf Intmed and Inf Ext of older animals (Fig. 5D). Scrutiny of

apoptosis densities within the GCL sublayers revealed an increasing gradient from the internal to the external third of the GCL ($F(2,17) = 381.4$, $p \leq 0.0005$; Fig. 5E).

BrdU and pHisH3-Ser10 positive cells

In contrast to apoptosis, there were no significant hemispherical differences in the densities of BrdU and pHisH3-Ser10 positive cells in any of the divisions of the dentate gyrus at 1 and 3 months of age (Fig. 7A).

The density of BrdU and pHisH3-Ser10 positive cells varied markedly within the various divisions of the dentate gyrus. As illustrated in Fig. 7B, significant differences were observed at both ages (1 month: $F(3,23) = 196.7$, $p \leq 0.0005$; 3 months: $F(3,15) = 137.1$, $p \leq 0.0005$). Interestingly, the relative degrees of cellular proliferation showed the following rank orders: SGZ >> GCL>> HI ~ ML and SGZ >> GCL ~ HI ~ ML, in 1- and 3-month old rats, respectively. With increasing age, there was a significant decrease in the density of proliferation in the GCL and SGZ, but an increase in the ML and HI (Fig. 7B).

Complementary analysis of differentiation markers in newly-proliferated cells showed that most cells had a neuronal phenotype (NeuN-positive) in the GCL and SGZ of 1-month old animals (Table 2). Such predominance was not so evident in the GCL and SGZ of 3-month old rats.

ANOVA showed significant differences in proliferation along the rostro-caudal axis of this hippocampal subfield ($F(3,23) = 44.4$; $p \leq 0.0005$). Post-hoc comparisons revealed that mitotic cells predominated in the ventral GCL and SGZ of younger animals (Fig. 6). Interestingly, the increase in volume between 1 and 3-month old animals was also higher in the ventral hippocampal divisions (Table 1). A more detailed analysis of proliferation at 5 equidistant points along the rostral-caudal axis (Fig. 7C) demonstrated a gradient of proliferation in both the GCL ($F(4,29) = 6.5$; $p = 0.001$) and SGZ ($F(4,29) = 14.9$; $p \leq 0.0005$). To confirm this observation, we examined the distribution of pHisH3-Ser10 staining at these 5 points. ANOVA revealed that the distribution of pHisH3-Ser10-positive cells differs in the GCL ($F(4,29) = 11.0$; $p \leq 0.0005$) and SGZ ($F(4,29) = 8.0$; $p \leq 0.0005$) along the dorsal-ventral axis of the hippocampus. Furthermore, correlation analysis (Pearson's) of the distribution of these two markers was significant for the GCL ($r2 = 0.509$, $p= 0.046$) and SGZ ($r2 = 0.672$, $p= 0.039$; Fig. 7D).

As illustrated in Fig. 6B, proliferating cells were differently located into supra- and infra-pyramidal blades of 1month old animals ($F(3,23) = 112.9$; $p \leq 0.0005$); post-hoc comparisons demonstrated an higher density of BrdU-labeled cells in the infra-pyramidal blade.

Analysis of the densities of BrdU-positive cells within the GCL and SGZ sub-divisions revealed a gradient of proliferation, increasing from the extreme portion of the suprapyramidal blade to the angular/intermediate portions of the infrapyramidal blade (Fig. 6B, 7E). Comparison between ages showed higher levels of proliferation in the Sup Intmed, Inf Intmed and Inf Ext sub-divisions of younger rats (Fig. 7E). Subdivision of GCL into external, intermediate and internal layers confirmed that proliferating cells are predominantly found in the internal portion of this layer (Fig. 7F).

Discussion

The postnatal dentate gyrus is one of the few brain areas that can generate new neurons throughout life. New granule neurons derive from progenitors cells located in the SGZ (Altman and Das, 1965; Cameron et al., 1993; Kuhn et al., 1996), adding to the volume and total cell number of the dentate gyrus (Sousa et al., 1998, 1999). However, the net increase in granule cell number in the mature organism is considerably smaller than that which would be predicted on the basis of established rates of cell proliferation (Biebl et al., 2000; Cameron and McKay, 2001); most likely, neurogenesis is counterbalanced by the concurrent demise of granule cells through apoptosis (Hassan et al., 1996; Heine et al., 2004). The programmed elimination of cells through apoptosis is important for the shaping, maintenance and reorganization of neural tissue (Raff et al., 1993; Burek and Oppenheim, 1996; Rubin, 1997). Hippocampal granule cells undergo natural apoptosis throughout development, but the rate of neurogenesis obviously exceeds that of cell death. Granule cells in the postnatal and mature organism are also subject to apoptosis, both natural and induced by a variety of exogenous factors, with their vulnerability increasing with advancing age (Gould et al., 1991; Sloviter et al., 1993; Gould and Cameron, 1996; Hassan et al., 1996; Almeida et al., 2000).

Many earlier studies suggested that the loss of hippocampal neurons (and reduced hippocampal volumes in pathological states) may, in part at least, contribute to impairments of mood and cognition as well as neuroendocrine dysregulation (Lupien et al., 1999; Honig and Rosenberg, 2000; Friedlander, 2003; Sheline et al., 2003; Sapolsky, 2004). As a corollary, a view that has gained momentum more recently is that neurogenesis in the hippocampus contributes to the improvements in mood observed following antidepressant therapy (refs). Neither of these claims has been supported by systematic studies of the contemporaneous rates of neurogenesis and apoptosis (cf. Gould and McEwen, 1993; Cameron and Gould, 1994; Lossi and Merighi, 2003). Also, none of the studies to date have considered the possibility that these events may be topographically overlapping (in which case, the gains and losses might balance out) or separate (when spatial differences in function could be an important issue). This study describes our observations on the topographical distribution of simultaneously-occurring neurogenesis and apoptosis in the juvenile and young adult rat; its results should serve to guide the design and interpretation of studies aimed to address the question: do the dynamics of granule cell

production and demise contribute to impaired behaviour and contribute to the efficacy of therapeutic remedies?

Our observations show that, in contrast to the distribution of proliferating granule cells, apoptotic cells were predominantly found in the suprapyramidal blade. While adrenalectomy-induced apoptosis was previously shown to mainly occur in this blade (Sloviter et al., 1993; Sousa et al., 1997), the current findings are the first to demonstrate such a skewed distribution under basal conditions, indicating the particular susceptibility of granule cells in this subdivision to apoptosis. Accordingly, these findings call for further studies into the significance of subregion-specific neuronal loss to the plasticity and function of the hippocampus. Although proliferation numerically exceeds apoptosis at younger ages, the latter, driven by changing internal and external conditions, is also likely to be an integral component of normal granule cell turnover, ensuring structural stability and appropriate connectivity.

Another striking result from our analysis was that apoptosis occurs to a greater extent in the left hippocampus. This finding bolsters the notion that differential granule cell elimination can have a significant impact on hippocampal structure: under basal conditions, standard morphometric and magnetic resonance imaging analyses revealed that the right hippocampus is thicker (Diamond et al., 1982) and has a greater volume than its left counterpart (Schubert et al., 2004; Verstynen et al., 2001). While lateralization of functions in the rodent brain is not clearly established, our results suggest that apoptosis may contribute to hemispherical asymmetries in hippocampal structure. However, it should be noted that asymmetry in hippocampal volumes in humans is well established; according to one study, up to 91% of healthy subjects have larger right hippocampal formations (Utsunomiya et al., 1999), and clinical data strongly indicate a higher incidence of certain psychiatric disorders (e.g. post-traumatic stress disorder and major depression) in subjects with smaller absolute and normalized left hippocampal volumes (Bremner et al., 1997; Mervaala et al., 2000). Further, (i) mood scores correlate negatively with left hippocampal volumes (Villarreal et al., 2002), and (ii) the normal left-right differences in hippocampal volumes are exaggerated in patients with affective disorders (Mervaala, et al, 2000). Interestingly studies in animals have shown that aversive early-life events, which have been causally implicated in psychopathology (Gutman and Nemeroff, 2003; McEwen, 2003), lead to a selective reduction in the size of the left hippocampus (Teicher et al., 2003). In light of these

observations, it seems imperative to consider apoptosis as an important factor in studies seeking to relate structural changes in the hippocampus with changes in mood and behavior.

The last assertion gains support if our analysis of the topographical profiles of neurogenesis (largely in agreement with previous reports - Kempermann et al., 2003) are considered in parallel with the profiles for apoptosis. While these two phenomena overlap in certain areas (both are greater in ventral than in dorsal dentate gyrus), they occur in distinct gradients along the various axes of the dentate gyrus (Fig. 6), raising questions about structural changes at precise anatomical levels with the function of those levels and their receptive fields. In summary, our investigation underlines the importance of considering the net loss or gain of neurons when evaluating the role of the hippocampus to neuro- and psycho-pathologies and the responses to treatment.

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Tables

Table 1. Volumes of the subdivisions of the dentate gyrus in the left and right hemispheres (mm3) of 1- and 3-months old rats.

		Left			Right		
Age		**Dorsal**	**Ventral**	**Total**	**Dorsal**	**Ventral**	**Total**
1 month	**ML**	2.15 (0.02)	1.59 (0.06)	3.74	2.03 (0.06)	1.48 (0.07)	3.51
	GCL	0.52 (0.01)	0.39 (0.02)	0.91	0.51 (0.02)	0.36 (0.02)	0.87
	SGZ	0.30 (0.01)	0.22 (0.01)	0.52	0.28 (0.01)	0.21 (0.01)	0.49
	HI	0.64 (0.03)	0.83 (0.04)	1.47	0.62 (0.01)	0.80 (0.04)	1.42
3 months	**ML**	3.05 (0.33)	3.13 (0.82)	6.17	3.03 (0.16)	3.21 (0.63)	6.24
	GCL	0.49 (0.06)	0.46 (0.12)	0.95	0.47 (0.01)	0.52 (0.1)	0.99
	SGZ	0.24 (0.02)	0.17 (0.02)	0.42	0.26 (0.03)	0.18 (0.02)	0.44
	HI	0.81 (0.12)	1.02 (0.20)	1,83	0.83 (0.02)	1.04 (0.24)	1.87

Values are expressed as means (+ SEM). Mean CE = 0.04. No significant differences were found between the values of left versus right subdivisions of the dentate gyrus at both ages. Comparisons between dorsal and ventral subdivisions of dentate gyrus in: (i) 1 month-old animals: ML; $p \leq 0.0005$; GCL: $p = 0.001$; SGZ: $p = 0.002$; HI: $p = 0.008$ and (ii) 3 month-old animals; SGZ $p = 0.018$. Age-comparisons showed that the volume of ML increased from 1 to 3 months of age ($p = 0.002$).

Table 2. Percentage of BrdU positive cells double-labeled with NeuN and GFAP in the dentate gyrus divisions of 1- and 3-month old animals.

	1month		3months	
	NeuN	**GFAP**	**NeuN**	**GFAP**
ML	37.5 (2.6)	37.3 (2.7)	33.6 (1.5)	44.7 (7.0)
GCL	53.3 (4.7)	8.3 (5.9)	32.8 (3.1)	24.3 (0.7)
SGZ	46.7 (2.6)	12.2 (4.2)	38.6 (1.5)	30.0 (0.8)
HI	47.0 (1.9)	50.0 (6.8)	22.4 (1.0)	16.9 (4.6)

Values are expressed as means (+ SEM). Neuronal differentiation predominates in the GCL and SGZ ($p \leq 0.001$) mainly in 1 month old animals. Comparison between ages showed a decrease % of cells undergoing neuronal differentiation at the time-point of analysis ($p \leq 0.004$); with increasing age the % of cells colocalizing BrdU+GFAP decreased in the HI ($p = 0.007$) but increased in the GCL and SGZ ($p \leq 0.06$).

Figures

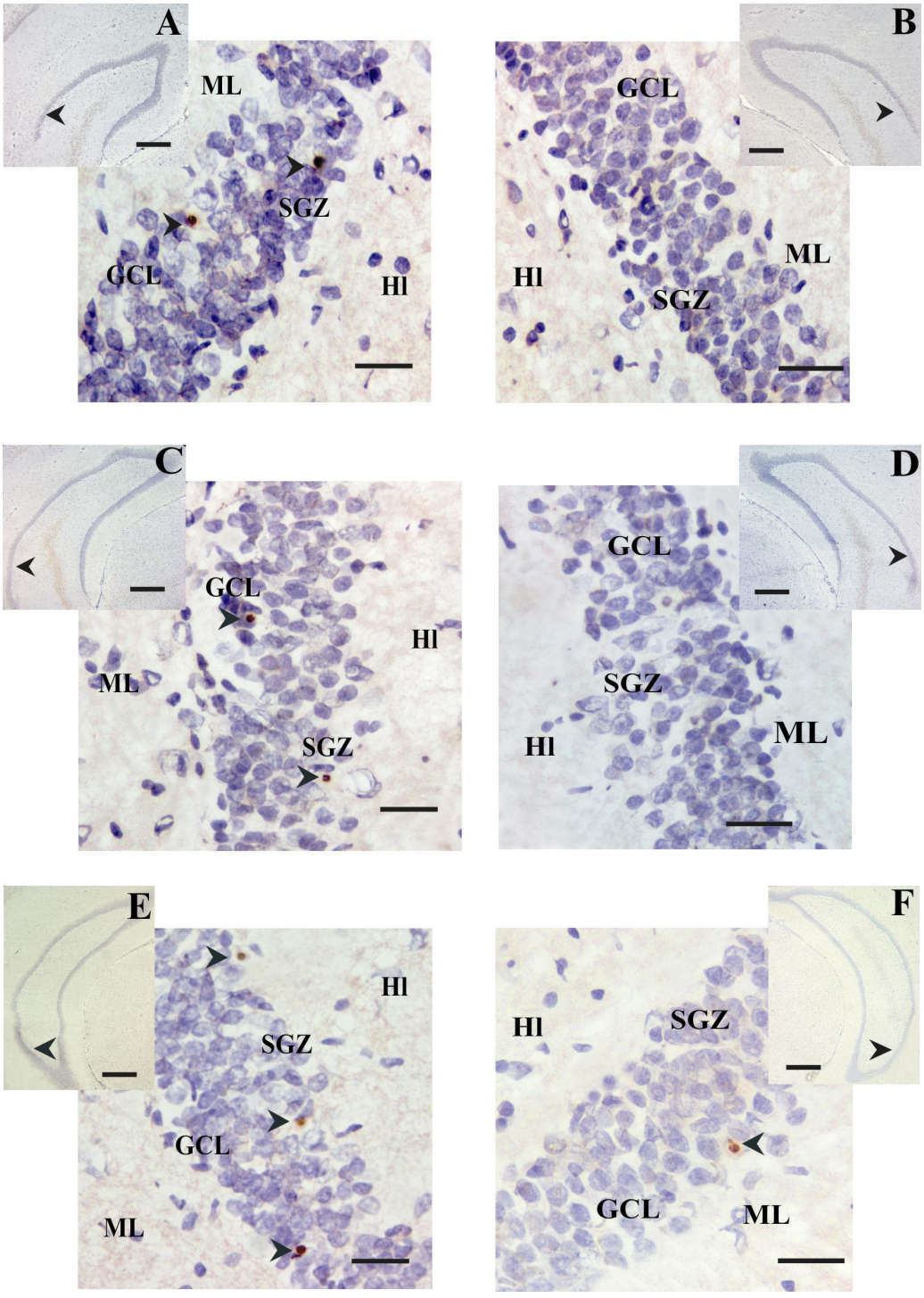

Fig. 1. Photomicrographs illustrating TUNEL positive cells (arrowheads) in the dentate gyrus along the rostro-caudal axis of the left and right hippocampus. Coronal sections of the dorsal (A to D) and ventral (E and F) dentate gyrus (scale bar for insets: 442 μm for A-D, 884 μm for E

and F) with a higher magnification from the extreme zone of each suprapyramidal blade (arrowhead; scale bar: 50 μm). A, C and E illustrate left hemispheres while B, D and F represent images from right hippocampal formations. Molecular layer (ML), granular cell layer (GCL), subgranular zone (SGZ) and hilus (HI).

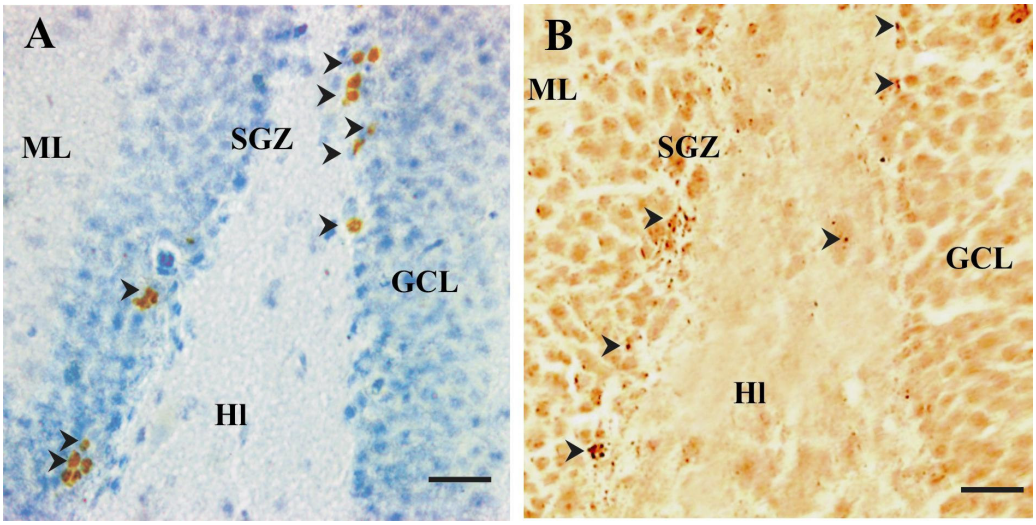

Fig. 2. Photomicrographs demonstrating the localization of (A) BrdU and (B) pHisH3-Ser10-immunoreactive cells (arrowheads). Molecular layer (ML), granular cell layer (GCL), subgranular zone (SGZ) and hilus (Hl). Scale bar: 80 μm.

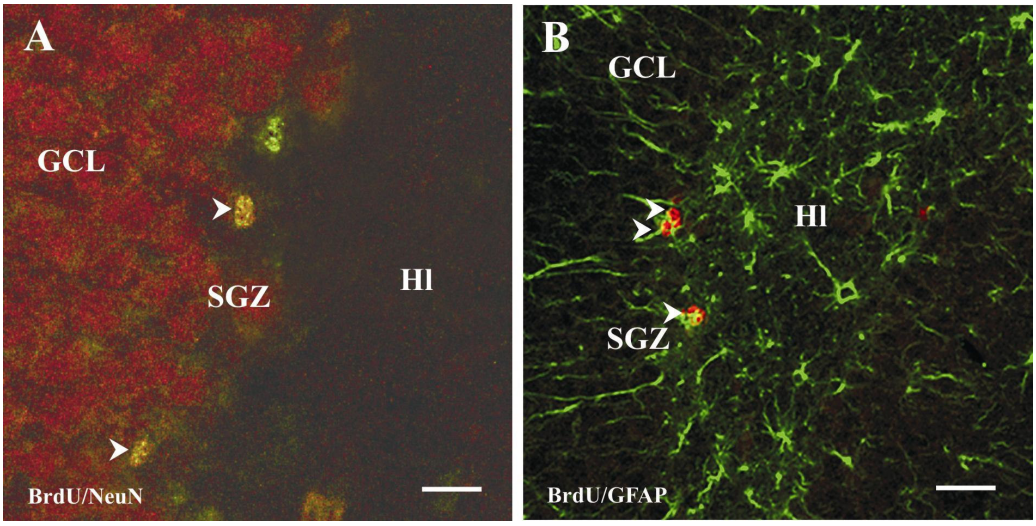

Fig. 3. Confocal photomicrographs illustrating colocalization of BrdU/NeuN and BrdU/GFAP. (A) Proliferating cells labeled with BrdU (green) and neuronal cells labeled with NeuN (red). Scale bar: 15 μm. (B) Proliferating cells labeled with BrdU (red) and glial cells labeled with GFAP (green). Arrowheads indicate double-labeling. Molecular layer (ML), granular cell layer (GCL), subgranular zone (SGZ) and hilus (Hl). Scale bar: 30 μm.

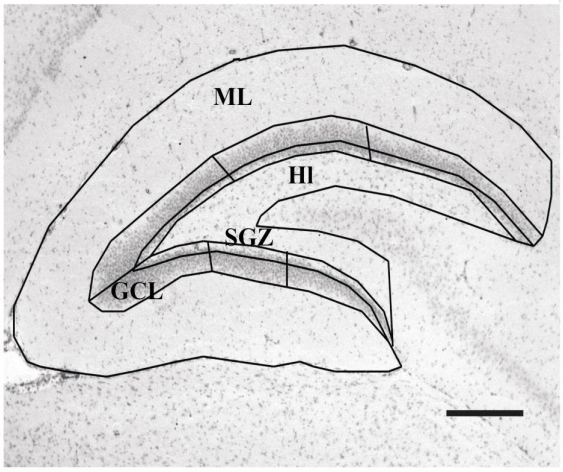

Fig. 4. Representation of the dentate gyrus divisions including the transversal subdivisions used for the cell counting with the StereoInvestigator software. Molecular layer (ML), granular cell layer (GCL), subgranular zone (SGZ) and hilus (Hl). Scale bar: 350 μm.

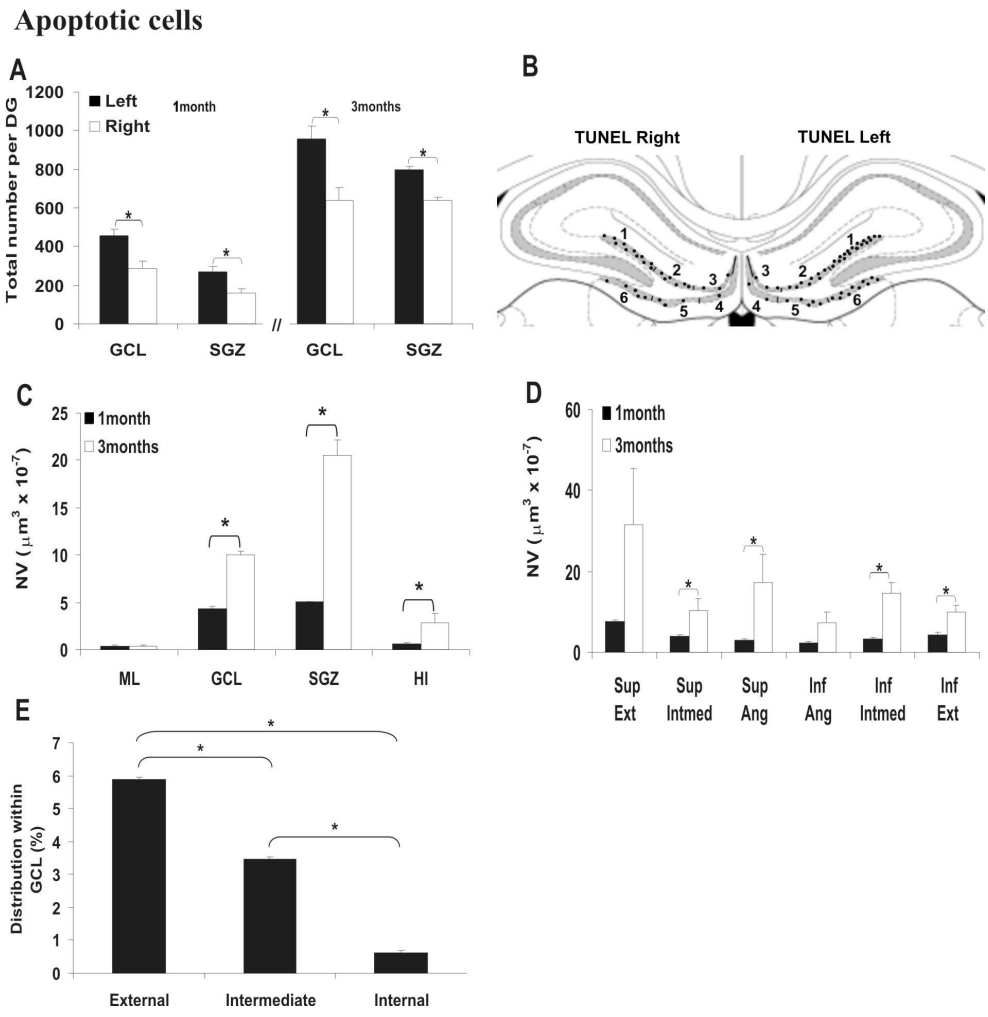

Fig. 5. Graphic representation of apoptotic cell distribution within the dentate gyrus (A) Comparison of total number of apoptotic cells in the left and right granular cell layer (GCL) and subgranular zone (SGZ) revealed a higher number of apoptotic cells in the left hemisphere at both ages (1 month: GCL; $p = 0.006$; SGZ. $p = 0.01$; 3 months: GCL: $p = 0.01$; SGZ: $p \leq 0.0005$). (B) Schematic illustration of the hemispheric differences in apoptosis indexes in the granule cell layer. (C) Graphic representation of the distribution of apoptotic cells in the molecular layer (ML), GCL, SGZ and hilus (HI) of 1 and 3 month old animals. At both ages, the SGZ displayed the highest apoptotic index, followed by the GCL (GCL vs ML; GCL vs SGZ; GCL vs HI; SGZ vs ML; SGZ vs HI: $p \leq 0.001$). Compared to the younger group, older animals had higher levels of apoptosis in the GCL and SGZ ($p \leq 0.0005$) and HI ($p = 0.02$). (D) Analysis of apoptotic cells density in the transversal subdivisions of the GCL and SGZ of 1 and 3 month old animals. The superior external subdivision had the highest levels of apoptosis ($p \leq 0.005$). Older animals

have higher levels of apoptosis than younger animals in the Sup Intmed ($p = 0.03$), Sup Ang ($p = 0.03$), Inf Intmed ($p \leq 0.0005$) and Sup Ext ($p = 0.05$). (E) Analysis of apoptotic cell levels in the longitudinal subdivisions of the GCL indicated an external-to-internal-layer gradient with higher levels of apoptosis occurring in the GCL external layer ($p \leq 0.0005$). *$p \leq 0.05$.

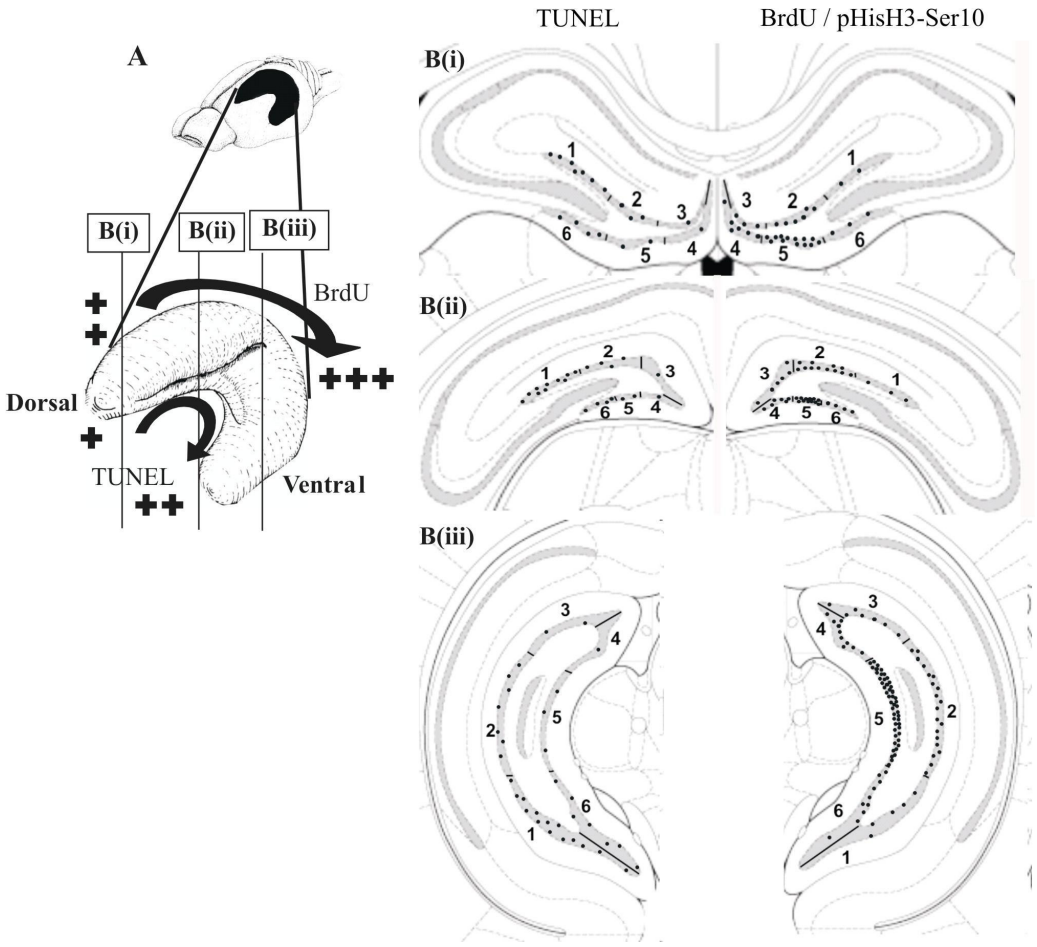

Fig. 6. Schematic comparison of the distribution of the density of apoptotic vs proliferating cells in the rat dentate gyrus. (A) The ventral division of the dentate gyrus displays higher rates of apoptosis and proliferation than the dorsal division ($p = 0.02$). (B) Illustration of the density of TUNEL vs BrdU/pHisH-Ser10 positive cells in the subdivisions of the GCL and SGZ of the hippocampal dentate gyrus (1: Sup Ext; 2: Sup Intmed; 3: Sup Ang; 4: Inf Ang; 5: Inf Intmed; 6: Inf Ext), in three positions along the rostro-caudal axis (i, ii and iii). The suprapyramidal subdivisions had a higher density of apoptosis than the infrapyramidal subdivisions (1 month GCL: $p= 0.005$; 3 month SGZ: $p = 0.01$), while proliferation predominated in the latter (1 month: GCL and SGZ $p \leq 0.0005$; 3 month: SGZ $p = 0.007$). A contrasting gradient of both processes along the tranversal divisions of the dentate gyrus could also be depicted: while apoptosis increased from Inf Ext to Sup Ext (6 to 1), the opposite occurred for proliferation.

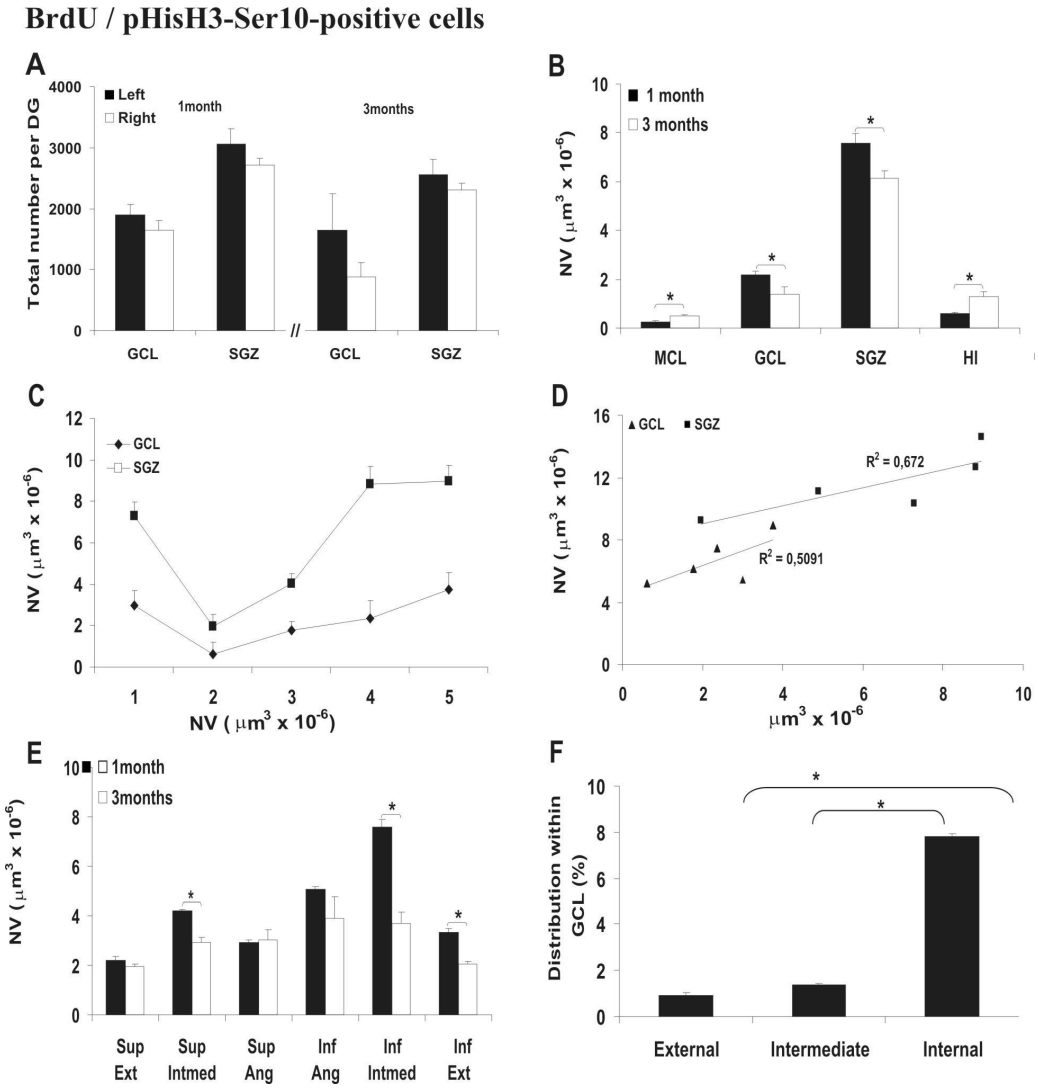

Fig. 7. Graphic representation of the distribution of the proliferating cells within the dentate gyrus. (A) iTotal number of BrdU-positive cells in the left and right granular cell layer (GCL) and subgranular zone (SGZ). (B) Density of BrdU-positive cells in the molecular layer (ML), GCL, SGZ and hilus (HI) of 1 and 3 month old animals. The SGZ displayed the highest levels of proliferation Age comparisons revealed that younger animals had higher levels of proliferation in the GCL ($p = 0.04$) and SGZ ($p = 0.03$) but lower densities in the ML ($p = 0.007$) and HI ($p = 0.006$). (C) Graphic representation of the distribution of BrdU positive cells in the dentate gyrus along 5 equidistant points along the rostral-caudal axis of 1-month old animals. There were significant differences of proliferation along this axis (GCL subdivisions: 1 vs 2, 5 vs 2, and 5 vs 3, $p < 0.05$; SGZ subdivisions: 1 vs 2, 5 vs 2, 5 vs 3, 4 vs 2, and 4 vs 3, $p < 0.05$). (D) Scatter plots

illustrating the correlation between the distribution of BrdU and pHisH3-Ser10 positive cells along the rostro-caudal axis of the GCL and SGZ. (E) Analysis of the densities of BrdU-positive cells between transversal subdivisions of the GCL and SGZ revealed Inf Intmed as having the highest levels of mitosis; this sub-division is different from all the others at 1 month old rats ($p \leq 0.001$) but in 3-month old animals the InfAng is different from Sup Ext ($p = 0.05$). Younger animals to have higher levels of proliferation in the Sup Intmed, Inf Intmed and Inf Ext sub-divisions ($p \leq 0.02$). (F) Distribution of BrdU-incorporating cells within longitudinal subdivisions of the GCL. The highest levels of mitosis levels were found in the internal sublayer (External vs Internal and Intermediate vs Internal: $p \leq 0.0005$). * $p \leq 0.05$

Chapter 2.2

Silva R, Mesquita AR, Bessa J, Sousa JC, Sotiropoulos I, Leão P, Almeida OFX and Sousa N

Stress-induced alterations in hippocampal neurogenesis and apoptosis are prevented by lithium

(Under review in Neuroscience)

Stress-induced alterations in hippocampal neurogenesis and apoptosis are prevented by lithium

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Abbreviations: BAG-1: B-cell-CLL/lymphoma2-associated athanonege; Bcl_2: B-cell CLL/lymphoma 2; BSA: bovine serum albumine; BrdU: bromodeoxyuridine; Cont: control; CMS: chronic mild stress; DAB: diaminobenzidine; DCX: doublecortin; FST: forced swimming test; GCL: granule cell layer; GFAP: glial fibrillary acidic protein; GR: glucocorticoid receptor; GSK-3: glycogen-synthase-kinase-3β (GSK3β); Hi: hilus; HPA-axis: Hypothalamic pituitary adrenal axis; HPRT: hypoxanthine guanine phosphoribosyl transferase; IMPase: inositol monophosphatase; ML: molecular layer; mRNA: messenger ribonucleic acid; NeuN: neuronal nuclei protein; PFA: paraformaldehyde; RT-PCR: reverse transcriptase polymerase chain reaction; Li: lithium; SEM: standard error of the mean; SGZ: subgranular zone; SVZ: subventricular zone; TBS: tris-buffer-saline; TUNEL: terminal transferase mediated dUTP nick-end-labeling.

Abstract

Mood disorders are the most common psychiatric disorders. Although the mechanisms implicated in the genesis of mood disorders are still unclear, stress is known to predispose to depression, and recently, studies have related hippocampal neurogenesis and apoptosis to depression. In the present study we first examined the balance between cell birth-death in the hippocampus and subventricular zone (SVZ) of pre-pubertal and adult rats subjected to chronic-mild-stress (CMS). CMS lead to increased corticosterone secretion and induced depressive-like symptoms (assessed in the forced-swimming test); these endocrine and behavioral effects were paralleled by decreased hippocampal, but not SVZ, cell proliferation/differentiation and by increased apoptotic rate. To determine if lithium, a known mood stabilizer with antidepressant properties, could prevent the stress-induced events, we analysed the same parameters in a group of rats treated with lithium during the stress exposure period (CMS+Li) and observed that the hormonal, behavioural and cell turnover effects of CMS were abrogated in these animals. Subsequently, to search for possible pathways through which CMS and lithium influence behaviour, cell fate and synaptic plasticity, we analysed the expression of glycogen-synthase-kinase-3β (GSK3β), B-cell-CLL/lymphoma2-associated athanonege (BAG-1) and synapsin-I. CMS increased GSK-3β and decreased synapsin-I and BAG-1 expression in the hippocampus. Interestingly, co-administration of lithium precluded the CMS-induced effects in GSK-3β, synapsin-I and BAG-1 expression. To further strength the involvement of the GSK-3β pathway in stress-induced effects, we showed that specific inhibition of this kinase with AR-A014418 blocked the effects of CMS in depressive like behaviour and in BAG-1 and synapsin-I expression.

In summary, these results reveal that lithium prevents stress-induced behavioral and cellular deleterious effects through regulation of molecular pathways altered by stress.

Key Words: Chronic mild stress, plasticity, glycogen synthase kinase-3β, Bcl_2 (B-cell CLL/lymphoma 2) - associated athanogene, synapsin-I.

Introduction

Depression is considered by the World Health Organization as a major contributor to the global illness burden at all ages, including childhood (Forbes and Dahl, 2005; Vitiello et al*.,* 2006). Chronic mild stress (CMS), one of the best validated animal models of depression (Wilner et al*.,* 2005), is also known to trigger deleterious effects in the hippocampus, including inhibition of proliferation (Czeh et al., 2001; Rosenbrock et al., 2005) and promotion of apoptosis (Lucassen et al., 2001). In addition, since hippocampal volumes are also reduced in depressive patients (for review see Sapolsky, 2000) a likely relationship between depressive states and the morphology of hippocampal formation has been proposed (Axelson et al., 1993). The hippocampal formation is a limbic structure, endowed with remarkable plastic properties that range from the morphological alteration of its dendrites (Sousa et al., 1999; 2000; Sousa and Almeida 2002) to the acquisition of new neurons in the subgranular zone of the dentate gyrus (Altman & Das, 1965; Eriksson et al., 1998; Silva et al., 2006).

Modulation of hippocampal neurogenesis has been proposed as a mechanism through which drugs regulating mood may act (Malberg et al., 2000). Here we assessed cell birth and death in an animal model of depression. Because our previous observations that neurogenesis and apoptosis do not occur uniformly in the dentate gyrus (Silva et al., 2006), we undertook a systematic stereology-based approach to analyze cellular proliferation and death in the dentate gyrus of the hippocampus of pre-pubertal and adult animals submitted to a CMS protocol. To establish if the effects were specific of the hippocampal formation, a similar analysis was performed in the subventricular zone, an area in which neurogenesis occurs prominently but one which is not associated with mood dysregulation.

Mood stabilizers, such as lithium were reported to prevent stress-induced alterations in hippocampal dendrite architecture (Wood et al., 2004) and in spatial memory performance (Vasconcellos et al., 2003) but not in anxiety behaviour (Wood et al., 2004). Interestingly, in the context of this study, lithium has also been reported to stimulate hippocampal neurogenesis in adult rodents (Chen et al., 2000). Lithium, the mainstay treatment for bipolar disease for many decades has many cellular actions, including the inhibition of glycogen synthase kinase 3α and β (GSK-3α/β) (Stambolic et al*.,* 1996) and inositol monophosphatase (IMPase) (Jope et al., 2003; Phiel & Klein 2001); in addition, chronic lithium treatment leads to an upregulation of glucocorticoid receptor (GR) mRNA expression in the hippocampus and hypothalamic paraventricular nucleus (Semba et al*.,* 2000), as well as upregulation of B-cell CLL/lymphoma 2 (Bcl_2) (Manji et al*.,* 2000a) and Bcl_2-associated protein-1 (BAG-1) (Zhou et al., 2005) expression in the hippocampus. Since a large proportion of depressed patients show cortisol hypersecretion, upregulation of GR seems to be one mechanism through which lithium may exerts its therapeutic actions. On the other hand, Bcl_2 and BAG-1 are implicated in neuronal survival, suggesting an alternative mechanism through which lithium can work to regulate mood. In order to assess if lithium treatment could prevent the CMS-induced effects in cell turnover we administrated lithium concomitantly to CMS and analysed the densities of cell proliferation and death in the hippocampal dentate gyrus and SVZ. Because neurogenesis and apoptosis share common molecular pathways, we also measured the hippocampal levels of GSK-3β after CMS and lithium administration; furthermore, a group of rats was treated with AR-A014418, a GSK-3β specific inhibitor, to ascertain the involvement of this kinase in CMS-mediated actions. Finally, synapsin-I and BAG-1, two downstream targets of GSK-3β, were analysed as endpoints of stress and lithium actions.

Materials and methods

Animals and treatment

One hundred and forty four male Wistar rats (Charles River, Barcelona, Spain) were used in this study; half of the animals were 4 week of age at the beginning at the experimental procedures. While the remaining were 2 month old. Animals were housed 3 per cage, under standard laboratory conditions (12h light cycle; 22° C, 55% humidity; food and water available *ad libitum*). Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation. Animals were divided into control (Cont) and chronic mild stress (CMS) groups. The CMS protocol (Willner et al., 2005) comprised a series of different insults that were changed daily, according to a weekly rotation plan (Table 1), for a period of 14 days (Fig. 1). During the same period subgroups of Cont and CMS rats were daily injected, intraperitoneally (i.p.), with lithium chloride (Li) (2.5 mEq/Kg body weight, Sigma, St. Louis, MO) or with the GSK-3β specific inhibitor AR-A014418 (30 μmol/Kg body weight, Sigma, St. Louis, MO) for 14 days (Fig. 1.1 and 1.2);. the remaining Cont and CMS animals received saline injections. Intraperitoneal administration of lithium was preferred to chow laced with lithium to assure consistent levels of the drug. All experimental groups were composed by 12 animals and were maintained on 0.9% sodium chloride in drinking water, in order to compensate for the loss of ions due to lithium administration. All animals received daily bromodeoxyuridine (BrdU) injections (50 mg/Kg body weight, i.p., Sigma, St. Louis, MO) during the last 3 days of the experimental period (Fig. 1).

Blood was collected from all animals at the end of the experimental procedure, immediately before brain sampling; the sampling took place between 3 and 5 p.m.. The mean plasma levels of lithium were 0.59 mmol/L (581 ± 5% of controls); importantly, animals receiving lithium did not present signs of illness (monitored by quality of fur, eye dryness and spontaneous locomotor

activity). Basal plasma levels of corticosterone were determined by radio-immunoassay (MP Biomedicals, Orangeburg, NY).

Forced Swimming Test

In order to confirm the depressive-like behaviour of the animals subjected to the CMS protocol, learned helplessness, a measure of susceptibility to depression-related behaviour, was analysed using the forced swimming test (FST). One day after the last stressors (confinement to a restricted space, tile cage and water deprivation) (Fig. 1) rats were placed in a cylinder filled with water (25° C), in such a way that they were compelled to swim without the aid of a solid support. Pre-test (10 min) was done 1 day before the test session (5 min); sessions were videotaped and latency to immobility (the time at which the animal stops swimming for the first time) and immobility times were computed by an investigator who was blind to the experimental details.

Tissue preparation

All animals were sacrificed by rapid decapitation (Fig. 1). For BrdU and TUNEL detection 6 brains were carefully removed, placed in cryoprotectant and snap-frozen in liquid nitrogen. Serial coronal sections (20 μm), extending over the entire length of the telencephalon, were obtained using a cryostat and mounted on poly-L-lysine-coated slides.

The remaining six brains were carefully removed being the hemispheres separated. The right hemispheres were post-fixed in 4% paraformaldehyde (PFA) for 48h, paraffin embedded, cut in coronal sections of 5 μm, mounted in non-coated glass slides and used for glycogen synthase kinase-3β (GSK-3β), synapsin-I, Ki-67 and doublecortin (DCX) immunohistochemistry. For mRNA

determinations, the hippocampi of left hemispheres were dissected out and snap-frozen in liquid nitrogen.

RNA extraction and semiquantitative RT-PCR

For GSK-3β and BAG-1 gene expression analysis total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA synthesis was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Semiquantitative PCR reactions were performed as previously described (Wong et al., 1994). Briefly, each PCR cycle included the following steps: 94°C for 30 s, 57 °C for 45 s and 72 °C for 60s. A sequential series of PCR reactions using each primer pair was initially run to determine optimal annealing temperature and cycle number to ensure amplification within the exponential phase of the amplification curve both for the gene under study and for the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT). The expression level of the reference gene (HPRT) was used as an internal standard, to which other PCR amplification products were normalized. HPRT was chosen as a reference gene since no variation in its expression was observed between groups. The oligonucleotide primers for GSK-3β, BAG-1 and HPRT were synthesized using the Primer3 software on the basis of the following GenBank sequences: NM_032080 (GSK-3β); NM_019133 (synapsin-I); NM_007860 (BAG-1); NM_012583 (HPRT). The sequences of oligonucleotide primers were: GSK-3β sense, TTGGAAATGGGTCATTTGGT; GSK-3β anti-sense, TCACAGGGAGTGTCTGCTTG; synapsin-I sense, CAGGGTCAAGGCCGCCAGTG; synapsin-I anti-sense, CACATCC TGGCTGGGTTTCTG; BAG-1 sense, ATGGAAACACCCTT GTCAGC; BAG-1 anti-sense, AAAACCCTGCTGGATGTCAG; HPRT sense GCAGACTTTG CTTTCCTTGG; HPRT anti-sense TCCACTTTCGCTGATGACAC. The values presented are the average of 3 PCR runs for each sample.

Immunocytochemical detection of mitosis, apoptosis and differentiation

Proliferation was assessed by immunocytochemistry (ICC) for BrdU incorporation (Fig. 2.1) and confirmed by Ki-67 (an endogenous restricted marker) (Fig 2.2). As previously described (Silva et al., 2006) BrdU positive cells were detected by ICC (mouse monoclonal anti-BrdU, 1:50, Dako, Glostrup/DK) in every 8th section For Ki-67 ICC representative sections of dorsal and ventral hippocampi were deparaffinized, immersed in TBS-T (0.1%) for 5 minutes and microwaved while immersed in citrate buffer (0.1 M) for 15 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 in TBS (10 minutes) and non-specific staining was blocked with 4% bovine serum albumin (BSA) in TBS (30 minutes). Subsequently, sections were incubated for 1.5 hours with a mouse monoclonal anti-Ki-67 antibody (1:200, Novocastra, Newcastle Upon Tyne, UK). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and diaminobenzidine (DAB: 0.025% and 0.5% H_2O_2 in Tris-HCl 0.05M, pH 7.2). Specimens were lightly counterstained with hematoxylin.

Apoptosis was estimated using morphological criteria and TUNEL histochemistry (Fig. 2.3) (TdT, BI Fermentas, Hanover/MD); dUTP-Biotin, Roche, Basel/Switzerland) in every 8th section, as previously described (Silva et al., 2006). In addition cell proliferation and death in the subventricular zone (SVZ) was estimated by measuring BrdU, Ki-67 and TUNEL positive cells in 16 sections evenly distributed along the hemispheric anterior-posterior axis.

The phenotype of newly-acquired cells was assessed by double staining of proliferation markers (BrdU and Ki-67) with differentiation markers (DCX for immature neurons, NeuN for mature neurons and GFAP for glial cells). Using the above described protocol a set of sections representative of the dorsal and ventral hippocampi were double stained for Ki-67 (mouse anti-Ki-67; 1:200, Novocastra, Newcastle Upon Tyne, UK) and DCX (rabbit anti-DCX polyclonal antibody, 1:500, Abcam, Cambrige/UK) (Fig. 2.4), while another set was stained for Ki-67 and

GFAP (rabbit anti-GFAP monoclonal antibody, 1:200, Dako, Glostrup/DK) (Fig. 2.5). The following secondary antibodies were used: anti-mouse Alexa Fluor-568 (Ki-67), anti-rabbit Alexa Fluor-488 (DCX and GFAP) (both1:200, Molecular Probes, Eugene/OR). One set of a 1-in-16 series of hippocampal sections was sequentially double stained for BrdU (rat anti-BrdU monoclonal antibody, 1:50, Abcam, Cambrige/UK) and NeuN (mouse anti-NeuN, 1:100, Chemicon, Temecula/CA) (Fig. 2.6), while another set was sequentially stained for BrdU (mouse anti-BrdU monoclonal antibody, 1:50, Dako, Glostrup/DK) and GFAP (rabbit anti-GFAP, 1:200, Dako, Glostrup/DK) (Fig. 2.7). The following secondary antibodies were used: anti-rat Alexa Fluor-488 (BrdU), anti-mouse Alexa Fluor-568 (NeuN), anti-mouse Alexa Fluor-568 (BrdU), anti-rabbit Alexa Fluor-488 (GFAP) (all 1:200, Molecular Probes, Eugene/OR).

For each animal, 50-100 BrdU or Ki-67-positive cells within the dentate gyrus were analyzed after double-staining with neuronal or glial markers, using Leica FT2 AOBS and Olympus FV1000 confocal microscopes.

Detection of GSK-3β and synapsin-I immunoreactivity

Glycogen synthase kinase-3 (GSK-3) and synapsin-I were detected by immunocytochemistry in sections representative of the dorsal and ventral hippocampi. Briefly, sections were deparaffinized and microwaved while immersed in citrate buffer (0.1 M) for 20 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 in TBS (10 minutes) and non-specific staining was blocked with 4% bovine serum albumin (BSA) in TBS (30 minutes). Subsequently, sections were incubated overnight with a mouse monoclonal anti-GSK-3β (1:100, Lab Vision, Fremont, CA) or mouse anti-synapsin-I (1:100, Sigma, St Louis, MO). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and diaminobenzidine (DAB: 0.025% and 0.5% H_2O_2 in Tris-HCl 0.05M, pH 7.2). Specimens were lightly counterstained with hematoxylin.

Quantification procedures

As previously described (Silva et al*., 2006*), proliferation and apoptotic densities were estimated in the different subdivisions of the hippocampus using the *StereoInvestigator* software (MicroBrightField, Williston/VT) and the optical fractionator method with a sampling size area of 350 x 350 μm for the molecular layer and the hilus or 100 x 150 μm for the granule cell layer and subgranular zone. The area of the counting frame was 30 x 30 μm. For topographic analysis, the hippocampal dentate gyrus was divided into its main subfields: molecular layer (ML), granular cell layer (GCL), sugranular zone (SGZ) and hilus (Hi). The granule cell layer and SGZ transversal divisions (angular, intermediate and extreme) were also studied. These subdivisions were further distinguished in terms of their position within the suprapyramidal and infrapyramidal blades. Longitudinal divisions of the GCL (internal, intermediate and external) and the division between dorsal and ventral hippocampus were also analysed.

In order to determine the density of proliferation and apoptosis of adult animals, and BrdU, Ki-67 and TUNEL positive cells in the SVZ of pre-pubertal rats, we used the *StereoInvestigator* software to draw the areas of interest and count the number of positive cells within those areas; results were expressed as number of cells per area.

To determine GSK-3β and synapsin I density, the *Alphalmager* program (AlphaInnotech, San Leandro, CA) was used. Densities were calculated by subtracting the optical density of the area of interest by the optical density of an adjacent area, thus eliminating background effects.

Statistics

The results are expressed as group means ± standard error of the mean (SEM). Coefficient of error (CE) was calculated accordingly to Gundersen & Jensen (1987). Statistical analysis was performed using SPSS 14.0 software (SPSS Inc., Chicago/IL). Prior to parametric analysis, the homogeneity of variance was confirmed by Levene's test. The effects of stress and lithium *per se* on hippocampal dentate gyrus final cell balance and on molecular pathways were assessed using independent-samples t-test, while the overall effects were studied through two-way analysis of variance (ANOVA). Differences in the densities of BrdU and TUNEL, between the left and the right hippocampal dentate gyrus were assessed by independent-samples t-test. Statistical significance was accepted when p was ≤ 0.05.

Results

Biometric, hormonal and behaviour markers of the efficacy of CMS and lithium treatments

In order to monitor the efficacy of our stress paradigm, three different biological parameters were measured: body weight gain, adrenal weight and plasma corticosterone levels. At both ages, stressed animals had a significant decrease in the body weight gain and an increase in the adrenal/body weight ($p \leq 0.05$) (Fig. 3.1 and 3.2); CMS also increased the plasmatic corticosterone levels ($p \leq 0.02$) (Fig. 3.3). Two-way ANOVA indicated that co-administration of lithium to CMS animals (CMS+Li) restored the adrenal/body weight and the plasmatic corticosterone levels ($p \leq 0.03$) (Fig. 3.2 and 3.3). Interestingly, lithium administration to non-stress rats increased the adrenal/body weight at both ages ($p \leq 0.05$) (Fig. 3.2).

The forced-swimming test was used to monitor depressive-like behaviour. Exposure to CMS increased immobility time and latency to immobility in the FST ($p \leq 0.003$) (Fig. 4.1 and 4.2). ANOVA showed that lithium co-administration (CMS+Li), in pre-pubertal as well as in adult animals, prevented the CMS-induced effects, increasing latency and decreasing immobility times ($p \leq 0.05$) (Fig. 4.1 and 4.2); paradoxically, lithium administration to stress-free rats increased immobility time when compared to controls ($p \leq 0.005$) (Fig. 4.1 and 4.2). Interestingly, administration of the GSK-3β specific inhibitor AR-A014418 to CMS and stress-free animals triggered antidepressant effects ($p \leq 0.05$) (Fig. 4.3 and 4.4).

In summary, these data demonstrate the efficacy of the CMS protocol herein used, as triggered increased corticosterone secretion and signs of depressive-like behaviour; these behaviour effects were blocked by lithium or AR-A014418 co-administration, which demonstrates that GSK-3β inhibition plays a role in depression like behaviour.

Lithium prevented CMS-induced decrease of hippocampal granule cell turnover

There is substantial evidence that depressive disorders are associated with morphometric abnormalities, including changes in the number/size of neurons and glia in discrete brain areas (for review see Sapolsky, 2000). As lithium influences multiple pathways related to cell birth, survival, maturation or death in different cell types and brain areas (Chen *et al.,* 2000; Chuang *et al.,* 2002; Manji *et al.,* 2000a), it becomes interesting to examine if this drug affects neurogenesis, differentiation and apoptosis also in a stressful context (CMS). As the hippocampal function is known to differ along its different axis (Moser and Moser, 1998), the topographic distribution of cell proliferation and death in the hippocampal dentate gyrus was systematically determined.

In pre-pubertal, rats CMS induced a decrease (except in the ML) of cell proliferation (BrdU and Ki-67) ($p < 0.004$), while lithium administration to stress-free animals increased cell proliferation ($p < 0.001$) (Fig. 6.1 and 6.2). Two-way ANOVA revealed that lithium co-administration (CMS+Li) precluded the CMS effects in cell proliferation ($p \leq 0.05$) (Fig. 5.1 and 5.2). Moreover, ANOVA showed an interaction between lithium and CMS in cell proliferation ($p \leq 0.0005$).

Analysis of TUNEL staining revealed that both CMS and Li increased the apoptotic densities in all areas of the hippocampal dentate gyrus ($p \leq 0.04$), except in the GCL of CMS animals (Fig. 5.3). Two-way ANOVA indicated that concomitant administration of lithium to stressed animals prevented the CMS- and lithium-induced effects in all areas of the hippocampus ($p \leq 0.001$) and revealed a positive interaction between stress and lithium in this action ($p \leq 0.0005$), as it inverted the individual action of each of these factors (Fig. 5.3).

Analysis of the combined balance of proliferation and apoptosis showed a decreased cell turnover after CMS ($p \leq 0.0005$). In contrast, lithium increased the balance of cell proliferation *vs* death in

the SGZ and hilus ($p \leq 0.003$), but decreased in the GCL, when compared to controls (Fig. 5.4). In addition, two-way ANOVA revealed that co-administration of lithium (CMS+Li) precluded the CMS effects (Fig. 5.4).

In the subventricular zone (SVZ), the other main neurogenic region in the adult brain, CMS in pre-pubertal increased the density of apoptotic cells ($p \leq 0.0005$), without affecting cell proliferation (Fig. 5.5 – 5.7). On the other hand, lithium administration to stress-free animals increased both SVZ proliferation and apoptosis ($p \leq 0.007$), which led to a raise of the final cell balance ($p \leq 0.007$) (Fig. 5.5 – 5.7). ANOVA showed that administration of lithium concomitantly to CMS, not only increased SVZ proliferation but also prevented the CMS-induced augment in apoptosis, which restored the final cell turnover ($p \leq 0.0005$) (Fig. 5.5 – 5.7).

The analysis of cell proliferation and apoptosis in adult animals revealed similar results to the ones obtained in pre-pubertal rats both in the hippocampal dentate gyrus (Fig. 6.1 – 6.3) and SVZ (Fig. 6.4 – 6.6). CMS decreased cell proliferation in the dentate gyrus (Fig. 6.1) but not in the SVZ ($p \leq 0.03$); in contrast, apoptosis was bolstered by CMS in the hippocampus ($p \leq 0.04$) (6.2) and SVZ ($p \leq 0.0005$) (Fig. 6.5). Co-administration of lithium prevented CMS-induced effects both in proliferation and apoptosis ($p \leq 0.05$) (Fig. 6.1 – 6.6). Taking together, there was a decrease in hippocampal dentate cell turnover after CMS. All these effects were blocked by lithium co-administration to CMS animals, even though lithium administration to stress-free animals increased both cell proliferation and apoptosis.

The topographic analysis of proliferation and apoptosis revealed that almost all experimental groups displayed the previously described patterns (Silva et al., 2006), in particular with respect to the predominance of apoptosis in the left hemisphere ($p \leq 0.03$) (Fig. 7.1 and 7.2). There were exceptions to this general rule: Li-treated animals did not present hemispheric differences

for apoptosis (Fig. 7.2) and in CMS+Li and Li groups the standard gradients of proliferation and apoptosis in transverse sections of the GCL and SGZ were not observable (data not shown).

In summary, these morphological analysis of cell proliferation and apoptosis demonstrated that CMS impairs cell turnover in regions of the brain endowed with proliferative properties; these action occurred by combining an inhibitory action in cell proliferation but also by triggering cell apoptosis (specially in the SGZ). Interestingly, lithium co-administration prevented these CMS effects and stabilized cell turnover in the hippocampal dentate gyrus and in and SVZ.

CMS and lithium modulate differentiation of newly-acquired cells. The scrutiny of the percentage of proliferating cells (Ki-67 and BrdU) that double-labelled with DCX, NeuN and GFAP in the GCL and SGZ of the hippocampal dentate gyrus revealed similar results in pre-pubertal and adult animals. While CMS decreased cell differentiation of both lineages, lithium administration to stress-free animals promoted the differentiation of newly-acquired cells into neurons or glia ($p \leq 0.04$) (Fig. 8.1 and 8.2). ANOVA showed that co-administration of lithium to stressed animals (CMS+Li) precluded the CMS effects ($p \leq 0.002$) (Fig. 8.1 and 8.2). These results indicate that besides decreasing proliferation, CMS also diminished both neuronal and glial differentiation; in contrast lithium triggered an opposite effect increasing differentiation of newly-acquired cells and therefore blocking CMS arresting effects in cell differentiation.

Lithium administration prevented CMS-induced changes in GSK3β, synapsin-1 and BAG-1

Many preclinical and clinical studies have implicated glycogen synthase kinase 3β (GSK-3β) in depressive illness (for review see Gould et al., 2006); moreover, this kinase is a direct and indirect target of lithium (Stambolic et al, 1996). Importantly, herein we observed that CMS

increased GSK-3β expression in the hippocampal formation ($p \leq 0.02$) (Fig. 9.1 – 9.4). Two-way ANOVA revealed that lithium co-administration (CMS+Li) prevented the CMS effects ($p \leq 0.04$); furthermore, there was a positive interaction between stress and lithium both in mRNA and protein analysis ($p \leq 0.005$) (Fig. 9.1 – 9.4). Interestingly, co-administration of a GSK-3β specific inhibitor (CMS+AR-A014418) also prevented CMS-induced increase of GSK-3β expression ($p \leq 0.005$) (Fig. 9.5 and 9.6). It should also be noticed that administration of AR-A014418 to stress-free animals also decreased GSK-3β levels ($p \leq 0.007$) (Fig. 9.5 and 9.6).

GSK3β is implicated in impaired synaptic plasticity and cognition (see Manji et al., 2001). We therefore monitored the expression of a presynaptic marker, synapsin-I, a known target of GSK3β (Hall et al*.,* 2000; 2002) and demonstrated that both mRNA and protein hippocampal levels of synapsin-I were decreased by CMS ($p \leq 0.04$), while lithium *per se* did not affected any of these parameters (Fig. 10.1 – 10.4). However, two-way ANOVA indicated that concomitant administration of lithium to CMS precluded the CMS effects in synapsin-I ($p \leq 0.05$) (Fig. 10.1 – 10.4). Co-administration of AR-A014418 to stressed animals (CMS+AR-A014418) also prevented the CMS-induced effects in synapsin-I ($p \leq 0.0005$) (Fig. 10.5 – 10.6); in addition, AR-A014418 *per se* also increased synapsin-I expression ($p \leq 0.006$) (Fig. 10.5 – 10.6).

The influence of CMS and lithium in the anti-apoptotic pathway was also studied. At both ages, CMS decreased BAG-1 gene expression ($p \leq 0.005$), while lithium *per se* increased the mRNA levels of this protein ($p \leq 0.006$) (Fig. 11.1 and 11.2). Two-way ANOVA showed that co-administration of lithium (CMS+Li) prevented the decreased induced by CMS ($p \leq 0.0005$) (Fig. 11.1 and 11.2). Consistently, co-administration of AR-A014418 (CMS+AR-A014418) also precluded CMS effects in BAG-1 expression ($p \leq 0.0005$) (Fig. 11.3 and 11.4); furthermore, administration of AR-A014418 to stress-free animals also increased BAG-1 mRNA levels ($p \leq 0.001$) (Fig. 11.3 and 11.4).

Taken together these molecular data demonstrate that CMS alters hippocampal GSK-3β expression; as a result two downstream targets of this kinase implicated in cell fate (BAG-1) and synaptic function (synapsin-I) are modulated by CMS. Importantly, in good correlation with behavioural and morphological data, administration of lithium (a non-specific inhibitor of GSK-3β) or AR-A014418 (a specific inhibitor of GSK-3β) to stressed animals blocked these molecular actions of CMS.

Discussion

Stress and corticosteroids are known to decrease neurogenesis and increase apoptosis within the hippocampal dentate gyrus (Gould et al., 1991; Almeida et al., 2000; Crochemore et al., 2005). Importantly, both events have been putatively associated to depression (Santarelli et al., 2003; Lucassen et al., 2004; Fuchs et al., 2004). While the observation that mifepristone, a glucocorticoid antagonist with antidepressant effects, normalized corticosterone-induced reduction in neurogenesis (Mayer et al., 2006) favoured a link between hypercortisolemia, reduced neurogenesis and depression, other studies have questioned such association. In fact, some reports show that: i) hippocampal neurogenesis does not necessary relate to changes in corticosteroid levels (Czeh et al., 2002; Heine et al., 2004a), ii) apoptosis is decreased after chronic unpredictable stress (Heine et al., 2004b), iii) reduced proliferation is not correlated with the development of learned helplessness (Vollmayr et al., 2003) and iv) stem cell proliferation is not altered in depressive patients (Reif et al., 2006). The present observations that CMS induced a depressive-like behaviour, which was paralleled by hypercortisolemia and a decrease in hippocampal, but not SVZ, cell turnover, is thus of relevance for this dispute. Interestingly, our data also showed that stress arrested not only proliferation but also neuronal differentiation, as CMS animals presented a smaller percentage of mitotic cells co-expressing neuronal (NeuN and DCX) markers. The CMS-induced decreased glial differentiation is also of notice, as it was striker than that of neuronal differentiation; moreover, a growing body of evidence is emphasizing the role of glia cells in mood disorders (Lee et al., 2007).

Lithium is a mood stabilizer that also triggers antidepressant actions. In fact, we here demonstrated for the first time that lithium administration to pre-pubertal and adult CMS animals blocks the induction of depressive-like behaviour. This observation is in accordance with previous

reports showing that chronic lithium administration specifically reverses helplessness behaviour (Redrobe and Bourin, 1999). To further strength the link between hippocampal function, stress and lithium, it should be noticed that spatial (but not working) memory deficits (Vasconcellos et al., 2003) and the CA3 dendrites atrophy induced by stress can be attenuated by lithium administration (Wood et al., 2004).

Lithium it is also known to modulate both neurogenesis (Chen et al., 2000) and apoptosis (Chen and Chuang, 1999), as confirmed in this study. In fact, the results herein presented demonstrate that lithium administration during the CMS protocol prevented the stress-induced changes in the ratio of cell acquisition/loss in the hippocampal dentate gyrus because, as expected, the administration of lithium to stress-free rats positively influenced both cell proliferation and differentiation. Taking into account that age seems to influence the ability of lithium to bolster neurogenesis (Yu et al., 2003) it is relevant to note that we have observed a positive influence of lithium in neurogenesis both in pre-pubertal and adult rats. In parallel, in the present study we also demonstrated that lithium increased apoptosis in stress-free animals, as previously observed by other authors (Song et al., 2004; Tomasiewicz et al., 2006). Notwithstanding this pro-apoptotic effect of lithium, as a result of our combined analysis, we demonstrated for the first time that the final balance between cell proliferation and apoptosis was increased in all dentate gyrus areas, with the exception of the GCL. As lithium *per se* triggered signs of depressive-like behaviour, we suggest that the balance of cell acquisition/loss in the GCL, where mature granule cells reside, might be the most interesting parameter to correlate with mood behaviour.

How might lithium promote the balance of cell acquisition/loss? Lithium is known to inhibit GSK-3β, a kinase with multiple roles in cell function (Stambolic et al., 1996), whose expression was

recently found to be up-regulated by glucocorticoids (Sotiropoulos et al., 2006), and whose specific inhibition (AR-014418) triggers antidepressant effects (Gould et al., 2004; present study). Our observation that the specific inhibition of GSK-3β, similar to lithium, blocked the depressive actions of CMS, implicates this kinase in these events. Moreover, our observation that inhibition of GSK-3β increased the levels of the pre-synaptic marker, synapsin-I might indicate a role of this pathway in the modulation of synaptic plasticity. The present experiments showed also that lithium administration precluded not only CMS-induced upregulation of GSK-3β, but also CMS-induced reduction in synapsin-I expression in several hippocampal sub-fields, including the mossy-fiber-CA3 connection. These observations are in accordance with previous studies, which indicate a stress-inducing decrease of hippocampal synaptic contacts (Semba et al., 2000; Sousa et al., 2000) or of another pre-synaptic protein (synaptophysin) (Xu et al., 2004), and also support previous observations that lithium promotes neuroplasticity (Manji et al., 2001).

Our results indicate that lithium also exerts its stabilizing effects after neuronal insults, by modulating neurogenesis and apoptosis. These actions are mostly mediated by different downstream cascades, but it is of relevance to note that, as previously reported by others (Manji et al. 2000a; Zhou et al*.,* 2005), we showed that lithium administration to stress and stress-free animals up-regulated the mRNA expression levels of BAG-1. Additionally, we here show that specific inhibition of GSK-3β with AR-A014418 increased BAG-1 expression. BAG-1 is a protein that interacts, and amplifies the actions of the anti-apoptotic function of Bcl-2 (Takayama et al., 1995; Manji et al., 2000a), but also modulates glucocorticoid receptor (GR) activity through its co-chaperone functions (Schneikert et al., 1999). Thus, it seems that stress influences occur through pro- and anti-apoptotic pathways and lithium preventive effects operate, through inhibition of GSK-3β, in these same pathways. Despite these actions we cannot exclude other

alternative pathways; indeed, many studies demonstrated that lithium presents several cellular effects, including the increase of the levels of brain-derived neurothrophic factor (BDNF) and cAMP response element binding protein (CREB) in the hippocampal dentate gyrus (Manji et al., 2000b; Fukumoto et al., 2001; D'Sa & Duman 2002).

The present experiments show that stress impairs the balance of cell acquisition/loss in the hippocampal dentate gyrus, but not in the SVZ. We also demonstrate the ability of lithium to stabilize mood behaviour in conditions of stress, by counteracting the stress-induced impairments in hippocampal cell turnover and synaptic plasticity. Interestingly, these effects appear to occur through the inhibition of GSK-3β and modulation of BAG-1 expression. These observations contribute to a better understanding the mechanisms underlying mood deregulation and unravel possible therapeutic targets to prevent stress-induced mood disorders.

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Tables

Table 1. Chronic mild stress protocol

	Monday	**Tuesday**	**Wednesday**	**Thursday**	**Friday**	**Saturday + Sunday**
Morning	Confinement to a restricted space	Exposure to empty bottle	Exposure to restricted food	Confinement to a restricted space	Exposure to empty bottle	↑
Afternoon	Damp Bedding	Tilted cage	Damp Bedding	Tilted cage	Damp Bedding	Reversed light/dark cycle over weekend
Overnight	Water deprivation	Food deprivation	Illumination	Water deprivation	Reversed light/dark cycle over weekend	↓

Figures

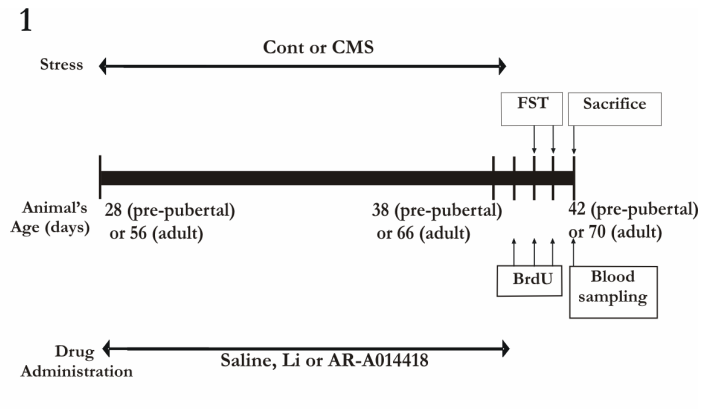

Fig. 1. Schematic representation of the time frame for chronic mild stress protocol (CMS), lithium administration, BrdU injections and forced swimming test (FST). Experimental procedures took place over a period of 14 days; during that period animals were injected with saline (Cont) or submitted to CMS; sub-groups of Cont and CMS rats received daily injections lithium (Li). All animals received 3 daily injections of BrdU in the last days of the experimental protocol. One day after the treatment procedures rats were submitted to FST. Twenty four hours after the behavioural assessment blood was collected and the animals were sacrificed.

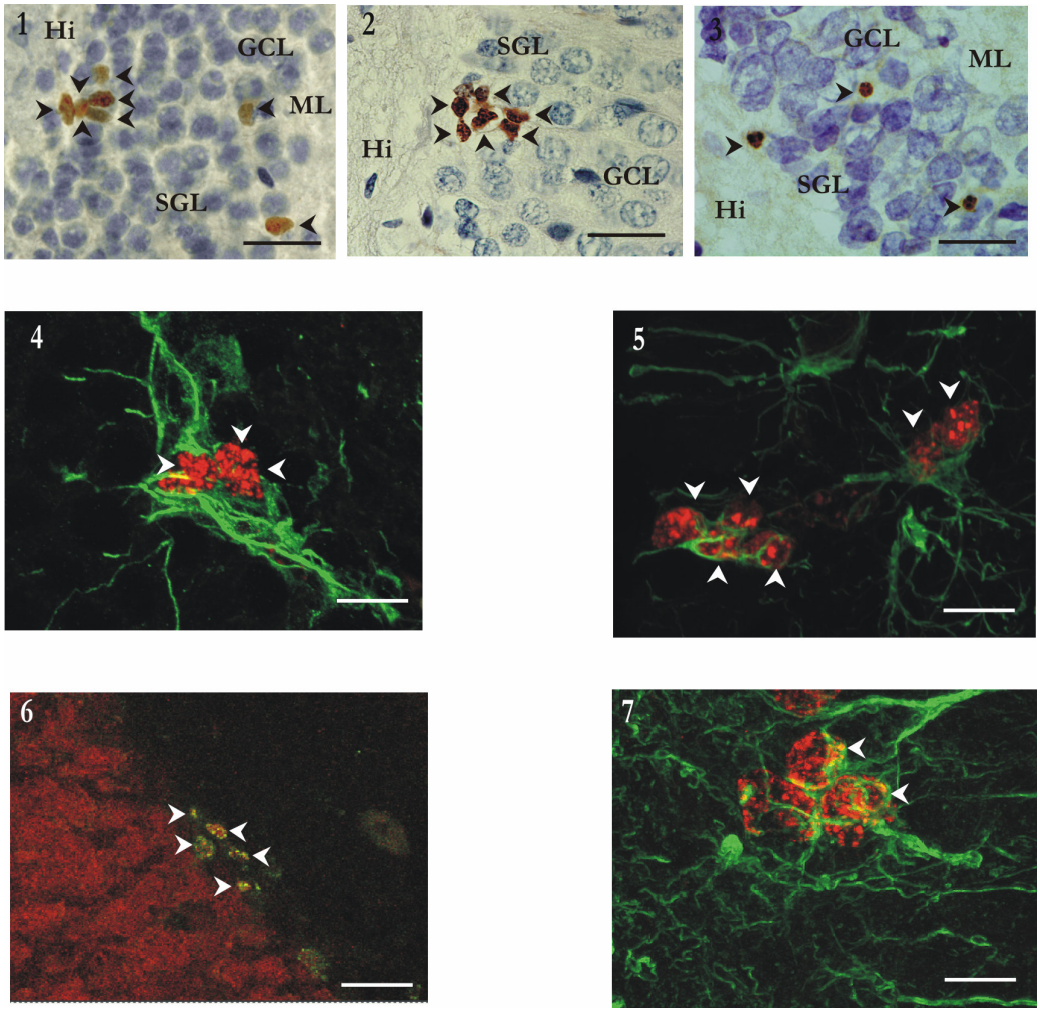

Fig. 2. Photomicrograph illustrating the localization of BrdU immunoreactive cells (Scale bar: 50 μm) **(2.1)**, Ki-67 immunostaining (Scale bar: 80 μm) **(2.2)**, and TUNEL positive cells (Scale bar: 80 μm) **(2.3**) in the hippocampal dentate gyrus (arrowheads). Molecular layer (ML), granule cell layer (GCL), subgranular zone (SGZ) and hilus (Hi). **(2.4)** Confocal photomicrographs illustrating colocalization of Ki-67 (red) / DCX (green) (scale bar: 15 μm). **(2.5)** Colocalization of Ki-67 (red) / GFAP (green) (scale bar: 15 μm). **(2.6)** Colocalization of BrdU (green) / NeuN (red) (scale bar: 30 μm). **(2.7)** Colocalization of BrdU (red) / GFAP (green) (scale bar: 10 μm). Arrowheads indicate double-labelling.

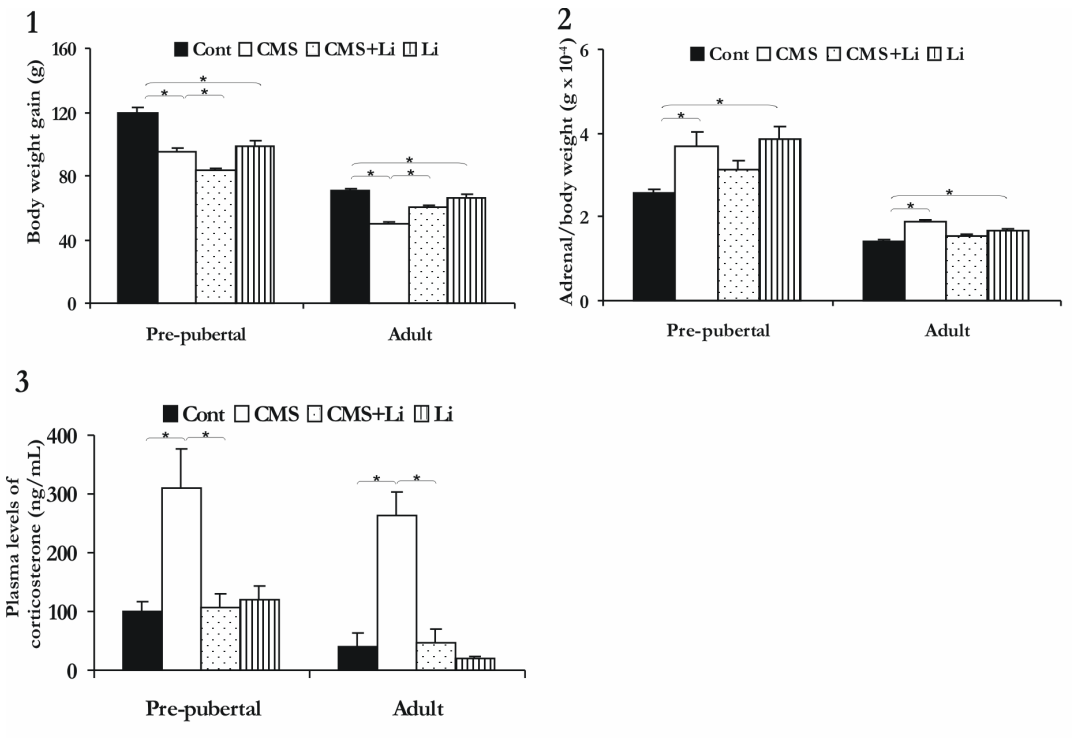

Fig. 3. Graphic representation of the comparison between control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) for biometric and hormonal markers in pre-pubertal and adult animals. At both ages, body weight gain **(3.1)** and adrenal/body weight **(3.2)** were, respectively, decreased and increased by CMS and Li ($p \leq 0.05$). CMS also increased corticosterone plasmatic levels ($p \leq 0.02$) **(3.3)**. Both in pre-pubertal and adult animals, CMS+Li prevented stress-induced actions in body weight gain and corticosterone plasmatic levels ($p \leq 0.03$) **(3.1 – 3.3)**.

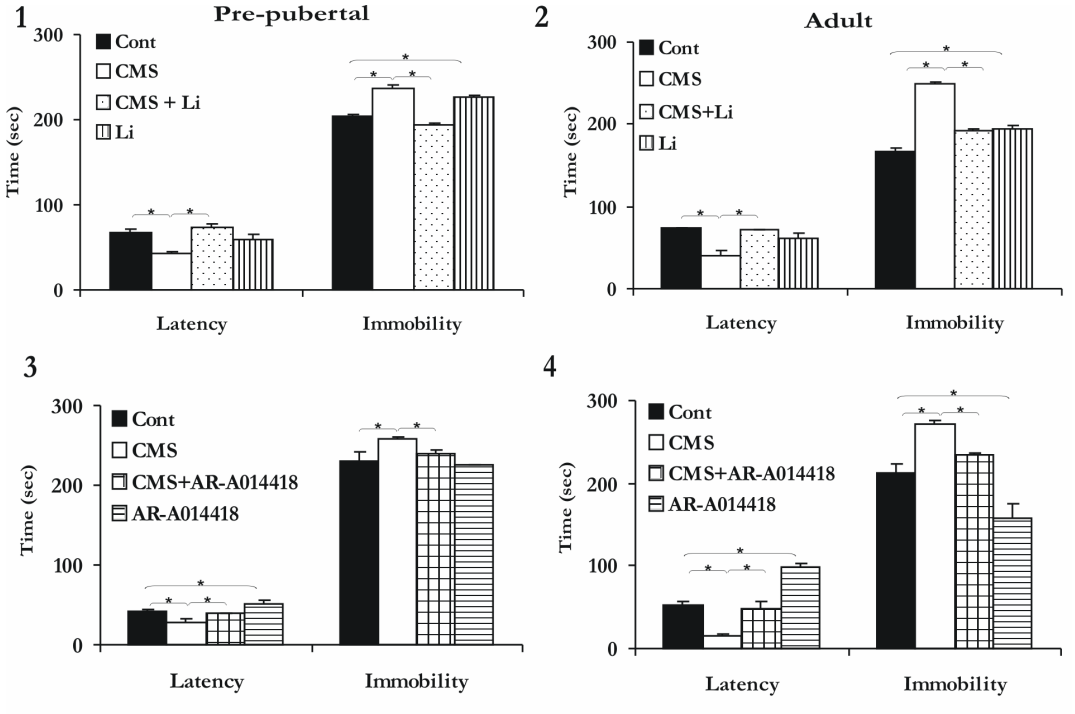

Fig.4. Graphic representation of the comparison between control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li), lithium (Li), chronic mild stress + AR-A01418 (CMS+AR-A014418) and AR-A014418 (AR-A014418) for the latency to immobility and immobility times in forced swimming test, in pre-pubertal and adult animals. CMS was observed to decrease latency to immobility and increase immobility times at both ages ($p \leq 0.0005$) **(4.1 and 4.2)**. However, the behavioural effects of CMS were prevented by concomitant administration of lithium at both ages ($p \leq 0.05$) **(4.1 and 4.2)**. Lithium administration to stress-free animals increased immobility time in pre-pubertal and adult animals ($p \leq 0.003$) **(4.1 and 4.2)**. Co-administration of AR-A014418 to stressed rats (CMS+AR-A014418) at both ages also precluded CMS behavioural effects ($p \leq 0.05$) **(4.3 and 4.4)**. AR-A014418 *per se* also triggered antidepressant effects by increasing latency to immobility and decreasing immobility times ($p \leq 0.04$) **(4.3 and 4.4)**.

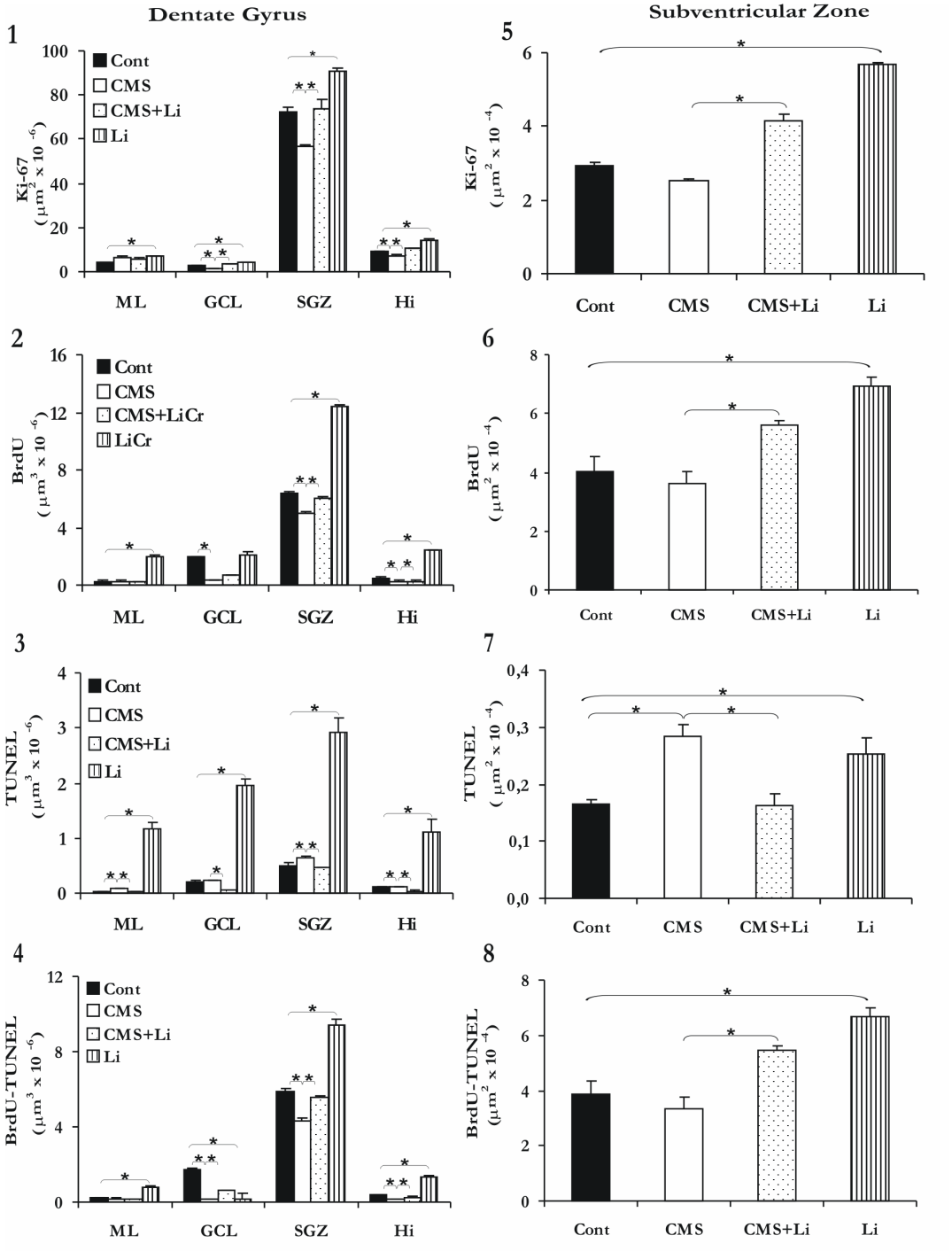

Fig. 5. Graphic representation of proliferation, apoptosis and the balance of cell acquisition-loss in the hippocampal dentate gyrus (DG) **(5.1 - 5.4)** and subventricular zone (SVZ) **(5.5 - 5.8)** of pre-pubertal control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) rats. There was a decrease in proliferation (Ki-67 and BrdU) in GCL, SGZ and Hi

(5.1 and 5.2) but not in SVZ **(5.5 and 5.6)** in the CMS group ($p \leq 0.004$); co-administration of lithium prevented the CMS-induced reduction in proliferation in the DG ($p \leq 0.0005$) **(5.1 and 5.2)**, and increased cell division in the SVZ ($p \leq 0.04$) **(5.5 and 5.6)**. The treatment with lithium in non-stress animals lead to an increase in proliferation, in the DG (except for GCL) and SVZ **(5.1 - 5.6)** ($p \leq 0.007$). Regarding apoptosis, there was a consistent increase in TUNEL positive cell density in the SVZ and dentate gyrus (except GCL) of CMS rats ($p \leq 0.04$), which was not observed in CMS+Li animals ($p \leq 0.04$) **(5.3 and 5.7)**. Lithium *per se* raised the density of apoptosis in the DG and SVZ ($p \leq 0.008$) **(5.3 and 5.7)**. The balance of cell acquisition-loss in the dentate gyrus was reduced by CMS. Lithium co-administration (CMS+Li) prevented the CMS effect in the DG and also induced an increase in the SVZ ($p \leq 0.04$) **(5.4 - 5.8)**. Lithium *per se* induced an increase of this parameter in DG (except in the GCL) and in SVZ ($p \leq 0.04$) **(5.4 - 5.8)**.

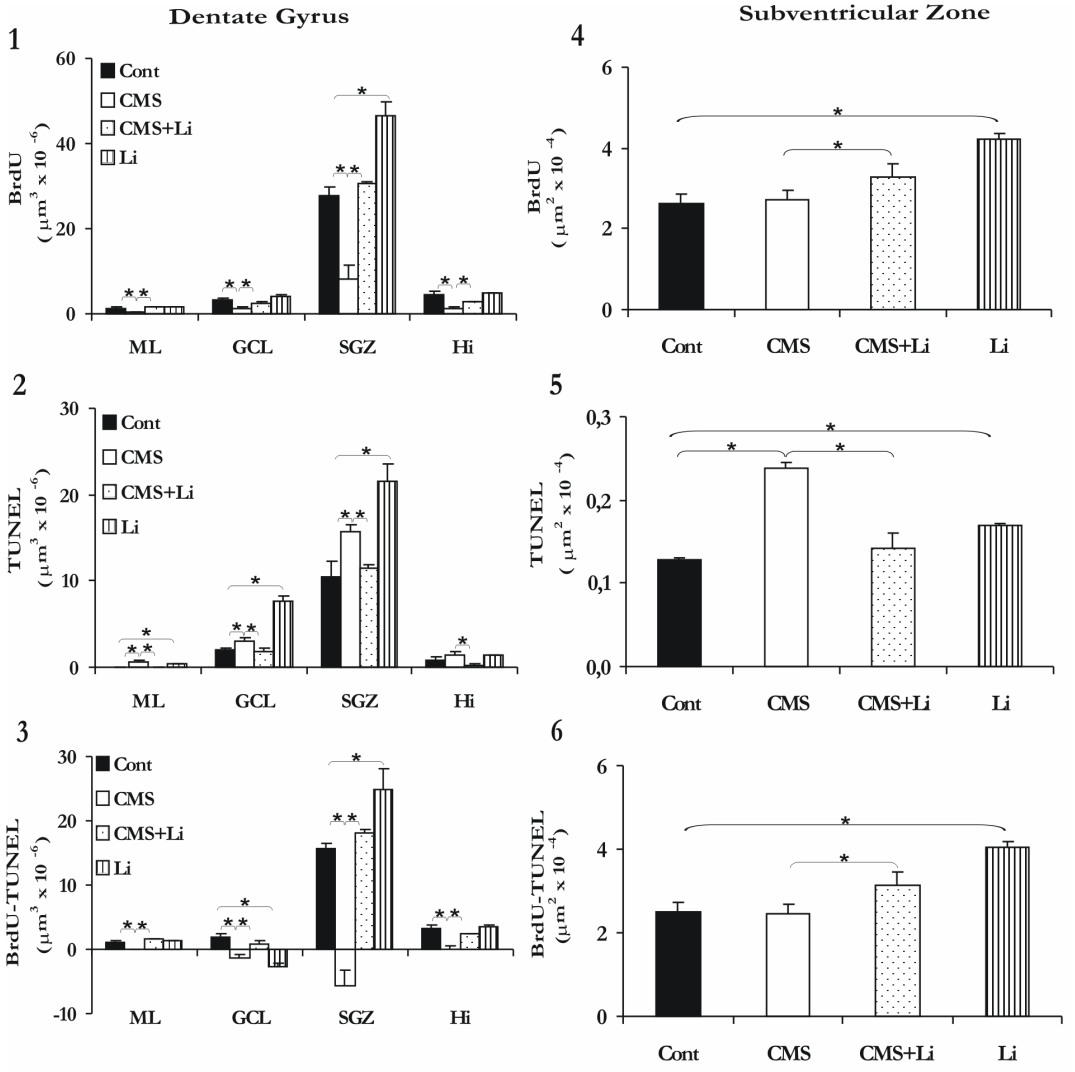

Fig. 6. Graphic representation of proliferation, apoptosis and the balance of cell acquisition-loss in the hippocampal dentate gyrus (DG) **(6.1 - 6.3)** and subventricular zone (SVZ) **(6.4 - 6.6)** of adult control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) rats. There was a decrease in proliferation in the DG **(6.1),** but not in SVZ **(6.4),** of CMS rats ($p \leq 0.03$); co-administration of lithium prevented the stress-reduction in proliferation in the DG ($p \leq 0.04$) **(6.1)**, and promoted proliferation in the SVZ ($p \leq 0.02$) **(6.4)**. Lithium administration to non-stress animals lead to an increase in proliferation in the SGZ **(6.1)** and SVZ **(6.4)** ($p \leq 0.001$). Regarding apoptosis, there was an increase in apoptotic cell density in the SVZ and dentate gyrus (except in Hi) of CMS animals ($p \leq 0.04$), which was not present in

CMS+Li rats ($p \leq 0.02$) **(6.2 and 6.5)**. Lithium *per se* raised the apoptotic density in the DG and SVZ ($p \leq 0.002$) **(6.2 and 6.5)**. The final balance of cell acquisition-loss was reduced by CMS in the dentate gyrus, but not in the SVZ ($p \leq 0.003$). Lithium co-administration (CMS+Li) precluded the CMS effect in the DG and induced an increase cell gain in the SVZ ($p \leq 0.04$) **(6.3 and 6.4)**. Lithium *per se* led to a decrease of cell gains in the GCL but an increase in the SGZ and SVZ ($p \leq 0.05$) **(6.3 and 6.4)**.

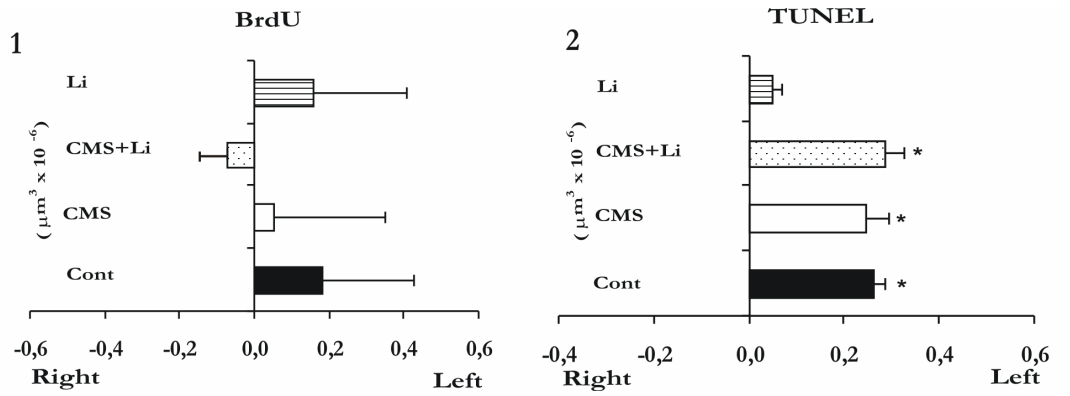

Fig.7. Graphic representation of the ratio between the densities of BrdU and TUNEL positive cells in the right/left hemispheres of the control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) groups. Although there are no significant hemispheric differences for cell proliferation, apoptosis is significantly higher in the left hippocampal dentate gyrus of Cont, CMS and CMS+Li groups ($p \leq 0.03$) **(7.1 and 7.2)**.

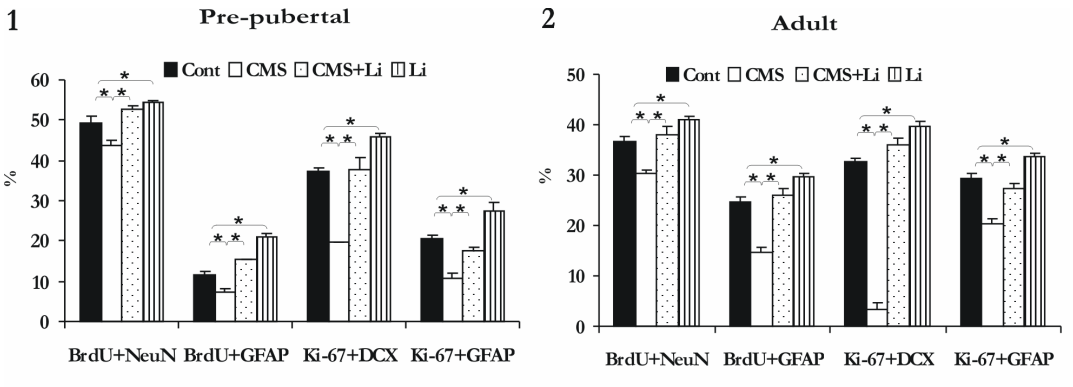

Fig.8. Graphic representation of the percentage of BrdU/Ki-67 positive cells that undergo differentiation in neuronal or glial lineage, in pre-pubertal and adult control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) groups. At both ages, CMS decreased neuronal (DCX and NeuN) and glial (GFAP) differentiation ($p \leq 0.04$), while administration of lithium to stress-free animals increased cell differentiation to both lineages ($p \leq 0.01$) **(8.1 and 8.2)**. CMS+Li prevented the stress-inducing effects in cell differentiation in pre-pubertal and adult animals ($p \leq 0.0005$) **(8.1 and 8.2).**

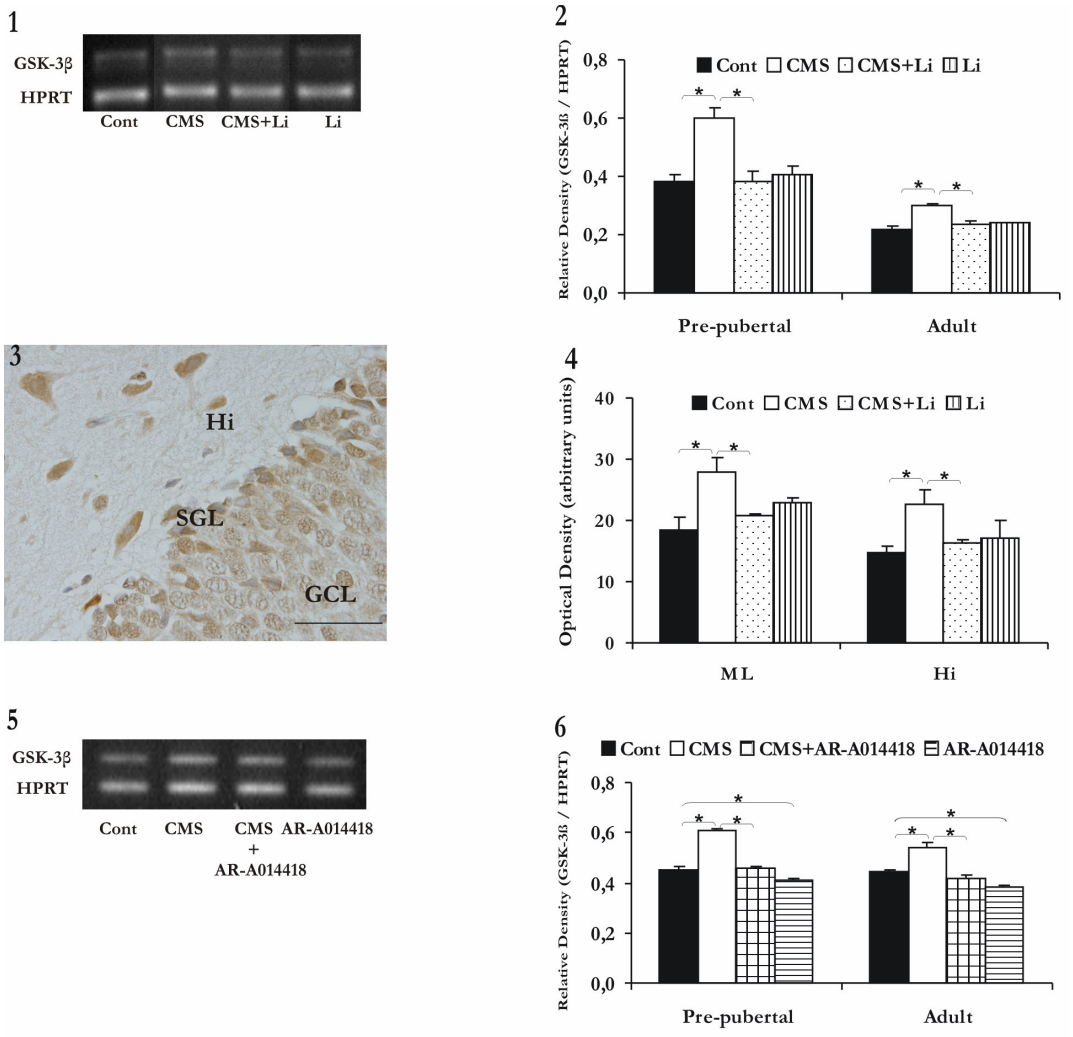

Fig. 9. Photomicrograph illustrating an example of a result from a hippocampal GSK-3β mRNA analysis **(9.1)**. Analysis of GSK-3β gene expression between control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) groups, showed increased levels of hippocampal GSK-3β in CMS, but not in CMS+Li group ($p \leq 0.003$), in pre-pubertal and adult **(9.2)** animals. The study of immunohistochemistry for GSK-3β **(9.3)** in the molecular layer (ML) and hilus (Hi) of the hippocampus of pre-pubertal rats corroborated the mRNA data, with the CMS-induced reduction of GSK-3β expressing cells being prevented by lithium co-administration ($p \leq 0.04$) **(9.4)**. The analysis, either by RT-PCR (both ages) or by imunohistochemistry (pre-pubertal), of the effects of lithium administration to stress-free animals, did not reveal alterations

in GSK-3β levels **(9.1 - 9.4)**. **(9.5)** Photomicrograph illustrating an example of a result from a hippocampal GSK-3β mRNA analysis between control (Cont), chronic mild stress (CMS), chronic mild stress + AR-A01418 (CMS+AR-A014418) and AR-A014418 (AR-A014418). This analysis showed that the CMS-induced increase of GSK-3β expression was prevented with AR-A014418 co-administration (CMS+AR-A014418) ($p \leq 0.0005$) **(9.6)**. Administration of AR-A014418 to stress-free animals also decreased GSK-3β mRNA levels ($p \leq 0.007$) **(9.6)**.

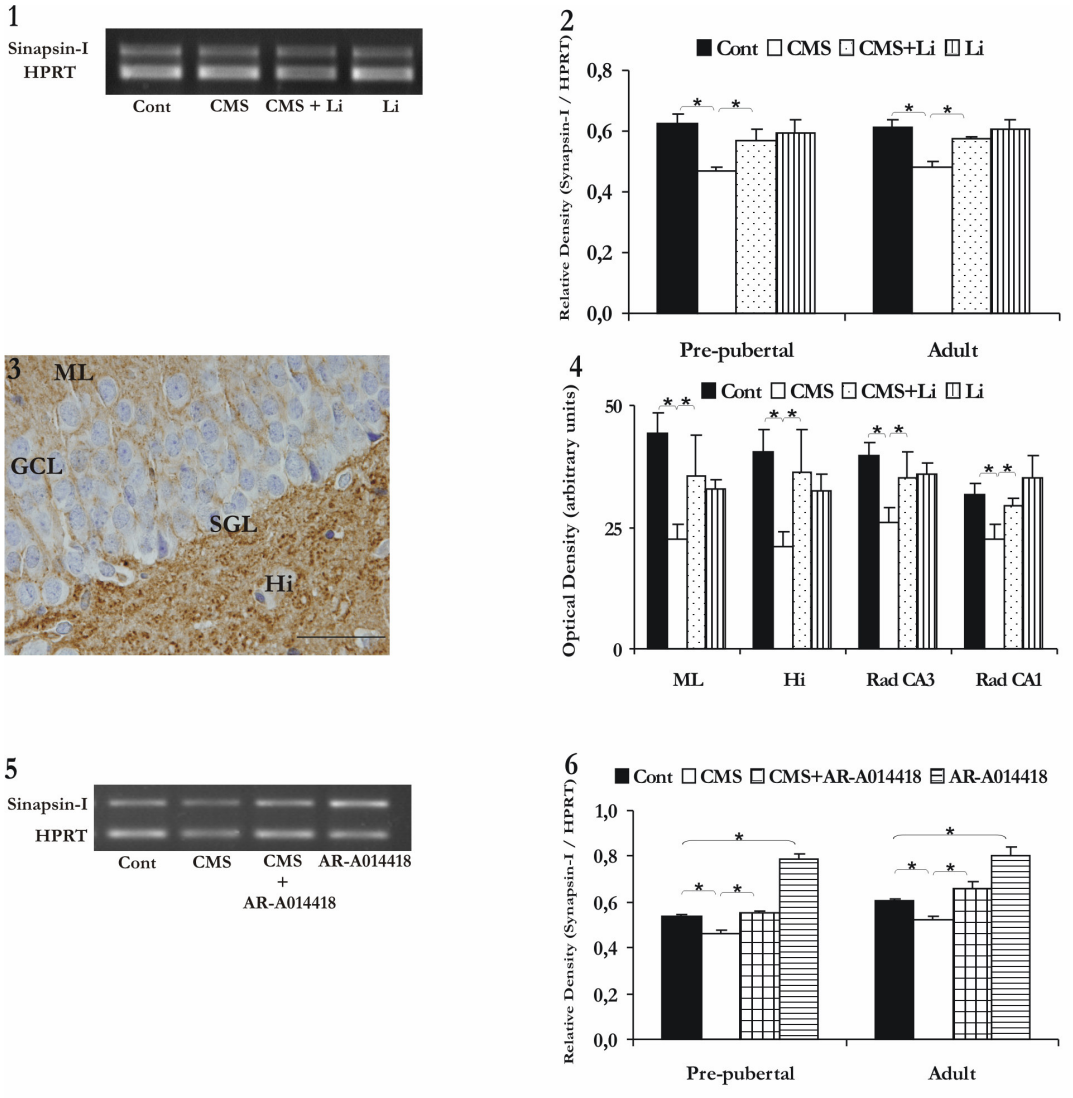

Fig. 10. Photomicrograph illustrating a synapsin-I mRNA analysis **(10.1)**. Comparison between control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) groups, showed a decrease in the hippocampal mRNA levels of synapsin-I in CMS, but not in CMS+Li or in Li groups, of both pre-pubertal and adult rats **(10.2)** ($p \leq 0.05$). Analysis in pre-pubertal animals of synapsin-I immunohistochemsitry **(10.3 and 10.4)** in the molecular layer (ML) and hilus (Hi) of the dentate gyrus and in radiatum layer of CA3 and CA1 fields supported the mRNA data by revealing that CMS, but not CMS+Li neither Li animals, displayed lower levels of synapsin-I than controls ($p \leq 0.04$) **(10.3 and 10.4)**. **(10.5)** Photomicrograph illustrating an

example of a result from a hippocampal synapsin-I mRNA analysis between control (Cont), chronic mild stress (CMS), chronic mild stress + AR-A01418 (CMS+AR-A014418) and AR-A014418 (AR-A014418). It was possible to observe that the CMS-induced decrease of synapsin-I expression was blocked by AR-A014418 co-administration (CMS+AR-A014418) ($p \leq 0.0005$) **(10.6)**. AR-A014418 per se also increased synapsin-I mRNA levels ($p \leq 0.006$) **(10.6)**.

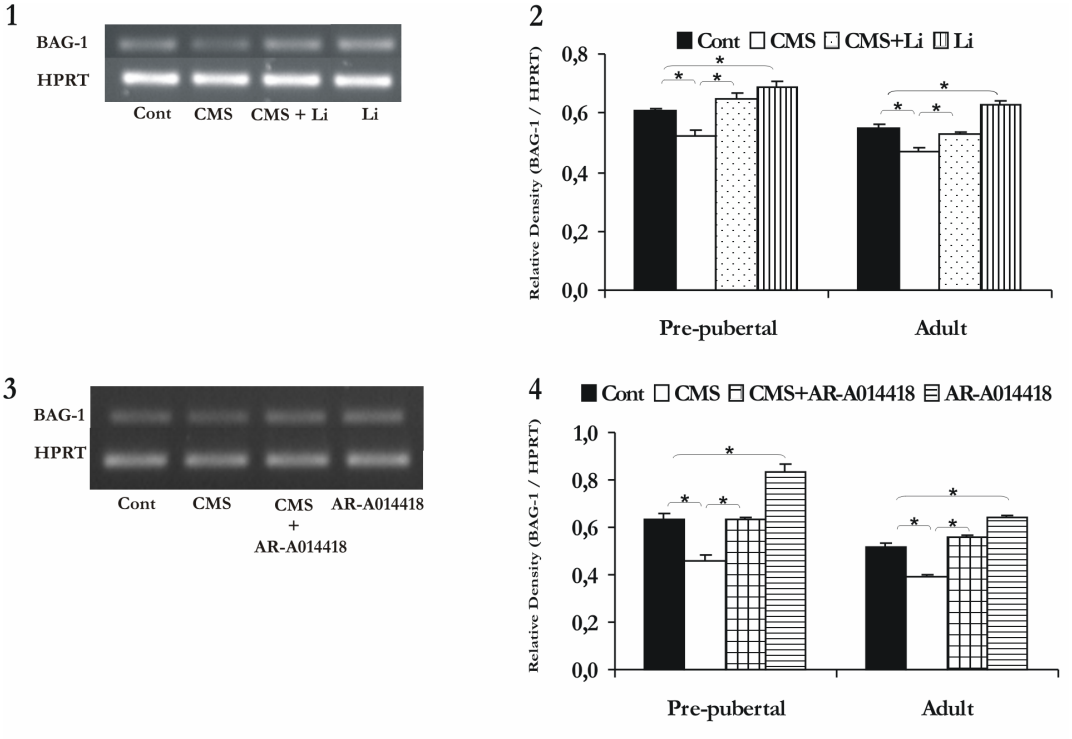

Fig. 11. Photomicrograph illustrating of a result of Bcl-2-associated athanogene protein-1 (BAG-1) hippocampal mRNA quantification **(11.1)**. Analysis of the BAG-1 levels between control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) in pre-pubertal and adult animals **(11.2)**, indicated a CMS-induced decrease in BAG-1 gene expression, which did not occur after lithium co-treatment (CMS+Li) ($p \leq 0.006$) **(11.1 - 11.2)**. Administration of lithium in stress free animals increased the mRNA levels of BAG-1 ($p \leq 0.005$) **(11.1 - 11.2)**. Photomicrograph illustrating an example of a result from a hippocampal BAG-1 mRNA analysis between control (Cont), chronic mild stress (CMS), chronic mild stress + AR-A01418 (CMS+AR-A014418) and AR-A014418 (AR-A014418) **(11.3)**. This analysis revealed that CMS-decrease BAG-1 expression ($p \leq 0.0005$), an effect that was prevented with AR-A014418 co-administration (CMS+AR-A014418) ($p \leq 0.0005$) **(11.4)**. AR-A014418 administration to stress-free animals also increased BAG-1 mRNA levels ($p \leq 0.006$) **(11.4)**.

Chapter 2.3

Silva R, Mesquita AR, Gonçalves L, Leão, P, Summavielle T, Almeida OFX and Sousa N The role of glycogen synthase kinase-3β in the modulation of depressive-like behavior and hippocampal cell turnover by corticosteroid milieu imbalances (In preparation)

The role of glycogen synthase kinase-3β in the modulation of depressive-like behavior and hippocampal cell turnover by corticosteroid milieu imbalances

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Abstract

Altered corticosteroid secretion has been reported in a significant percentage of patients suffering from depression. Corticosteroids are potent modulators of neuronal plasticity, namely in the hippocampal formation; besides triggering volumetric changes in the hippocampus, stress hormones are known to influence hippocampal neurogenesis and apoptosis. In the present study, we estimated the impact of corticosteroid imbalances in hippocampal cell turnover; to achieve this goal, we analysed rates of proliferation and apoptosis in rats treated with corticosterone (Cort), a ligand of both mineralocorticoid (MR) and glucocorticoid (GR) receptors, with dexamethasone (Dex) which only binds to GR, or submitted to adrenalectomy (ADX). Correlates between these parameters and depressive-like behaviour were established using the forced-swimming test. In addition, to scrutinize the ability of lithium (a mood stabilizer with antidepressant properties) to prevent the effects of corticosteroid imbalances in hippocampal cell fate, the same parameters were analysed in a replica of the above-described experimental groups that were co-treated with lithium. To explore the molecular pathways by which corticosteroids and lithium might act, we evaluated the expression of hippocampal glycogen synthase kinase-3β (GSK-3β), synapsin-I and B-cell-CLL/Lymphoma2-associated athanogene-1 (BAG-1).

All manipulations of the corticosteroid milieu triggered depressive-like behaviour and decreased the hippocampal dentate gyrus final cell balance; however, while Dex treatment increased GSK-3β expression and decreased synapsin-I and BAG-1 levels, ADX reduced the expression of all analysed proteins. Lithium co-administration prevented the behavioural and molecular effects of Dex; it also precluded the actions of ADX in behaviour, final cell balance and BAG-1 levels, but accentuated the effects of ADX in GSK-3β and synapsin-I expression.

In summary, these results reinforce the perspective that corticosteroids imbalances influence depressive-like behavior and strengthen its association to altered hippocampal cell turnover. In

addition, these events affect hippocampal GSK-3β expression, and some of its downstream targets, revealing a pathway through which lithium stabilizes mood under conditions of altered corticosteroid milieu.

Introduction

Corticosteroids are amongst the most powerful modulators of the structure and function of the central nervous system (CNS) (Sousa et al., 2007). As a consequence, these hormones are implicated in the aetiology of some brain pathologies, including depression (Gillespie and Numeroff, 2005). In fact, patients with Cushing's syndrome often experience severe depression and anxiety (for review see Sonino et al., 2001), while a significant number of subjects with depression present hypercortisolemia (Gibbons JL et al 1962; Axelson et al., 1993).

There are two distinct receptors for corticosteroids: mineralocorticoid (MR) and glucocorticoid (GR) receptors (Krozowski et al., 1983). These receptors are not homogeneously distributed in the brain (the hippocampal formation presents the highest density of both receptors - Ahima et al., 1990 and 1991) and display different affinity for corticosterone: while MR are largely activated with the basal levels of corticosterone, GR are mainly activated during the circadian peak or after exposure to stress (for review see de Kloet., 1990). Importantly, the impact of corticosteroids upon the CNS depends on the ratio of MR *versus* GR occupation (Sousa et al 2007). In terms of neuronal fate, while MR activation promotes neuroprotective mechanisms through the reduction of the Bax-Bcl-2 ratio and p53 levels (Almeida et al., 2000), the occupation of GR increases hippocampal apoptosis (Gould et al., 1991b), possibly through an increase of Bax/Bcl-2 ratio, p53 levels (Almeida et al., 2000) and glycogen synthase kynase-3β (GSK-3β) activity (Grimes and Jope, 2001; Smith et al 2002). Furthermore, besides increasing apoptosis, GR activation is also known to decrease hippocampal cell birth (Gould et al., 1999a).

Taking into consideration that depressive patients present a reduction of hippocampal volumes (Sapolsky 2000), a possible association between depression and the hippocampus structure has

been proposed. The hippocampus is a highly plastic brain region, which includes the generation of new neurons in the adult dentate gyrus (Altman and Das 1965; Eriksson et al 1998; Silva et al 2006). Moreover, neurogenesis in the hippocampus has been suggested as a mechanism through which classical antidepressants might act (Malberg et al 2000). While stress/corticosteroids are known inhibitors of adult neurogenesis, mood stabilizers, such as lithium, have been reported to stimulate hippocampal proliferation (Chen et al 2000). In parallel, corticosteroids, through GR activation, promote hippocampal apoptosis whereas lithium has been advanced as putative anti-apoptotic agent by promoting Bcl-2 and BAG-1 expression (Manji et al., 2000; Zhou et al., 2005). Since a significant proportion of depressed patients show hypercortisolemia, and considering that GR activation induces an upregulation of GSK-3β (Smith et al 2002; Sotiropoulos et al., 2006), the modulation of this enzyme by different status of corticosteroid receptors occupancy and by lithium (a inhibitor of GSK-3β (Stambolic et al., 1996) might prove to be a relevant key-player in the regulation of hippocampal cell fate. In addition, as molecular targets of the activity of GSK-3β, we measured the hippocampal expression of BAG-1 and synapsin-I.

Materials and methods

Animals and treatment

One hundred and forty four male Wistar rats (Charles River, Barcelona, Spain), aged 4 weeks were used in this study. Animals were housed under standard laboratory conditions (12/12h light/dark cycle; 22° C, 55% humidity; food and water available *ad libitum*). Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Experimental groups were composed by 18 animals each. Animals were divided in control (Cont), corticosterone (Cort), dexamethasone (Dex) and adrenalectomy (ADX) groups. Vehicle, corticosterone (40 mg/Kg body weight, Sigma, St. Louis, MO) or dexamethasone (0.3 mg/Kg body weight, Sigma, St. Louis, MO) were daily injected subcutaneously for 15 days. Subsets of 9 rats from each of these groups were co-treated with lithium chloride (Li) (2.5 mEq/Kg body weight, Sigma, St. Louis, MO) intraperitoneally (i.p.) during the same period. Bilateral ADX was performed, on the first day of the experimental period under ketamine (7.5g/100g body weight, i.p., Imalgene, Merial, Lyon-France) + medatomidine (50mg/100g body weight, i.p., Dormitor, Orion Pharma, Espoo-Finland) anaesthesia. 6 animals in each group received daily bromodeoxyuridine (BrdU) injections (50 mg/Kg body weight, i.p., Sigma, St. Louis, MO) during the last 3 days of the experiment. For replacement of ion lost due to lithium administration or ADX, all animals were maintained on 0.9% sodium chloride in drinking water; their body weight was recorded weekly.

Blood was collected from all animals at the end of the experimental procedure, 24 h after the last injection of corticoids and lithium. Basal plasma levels of corticosterone were determined by radio-immunoassay (MP Biomedicals, Orangeburg, NY). After

Forced Swimming Test

In order to evaluate the learned helplessness behaviour, a measure of depression-like behaviour, the forced swimming test (FST) was performed. Twenty four hours before sacrifice rats were placed in a cylinder filled with water (25° C) so that they were compelled to swim without the aid of a solid support. A 10 min pre-test was performed 1 day before the test session (5 min); sessions were videotaped and latency to immobility and immobility times were computed by an investigator who was blind to the experimental details. In the day in which the FST was performed animals were injected after the FST to avoid interference with the behavioural assessment.

Tissue preparation

Animals were sacrificed by rapid decapitation 24 hours after the last BrdU injection. Brains used for mRNA determinations, were carefully removed, the hippocampi were dissected out and snap-frozen in liquid nitrogen. Brains for BrdU and TUNEL detection were carefully removed, placed in cryoprotectant and snap-frozen in liquid nitrogen. Serial coronal sections (20 μm), extending over the entire length of the telencephalon, were obtained using a cryostat and mounted on poly-L-lysine-coated slides. Brains for GSK-3β, synapsin-I, Ki-67 and doublecortin (DCX) immunohistochemistry were removed, fixed in 4% paraformaldehyde (PFA) for 48h, paraffin embedded and cut in coronal sections (5 μm).

RNA extraction and semiquantitative RT-PCR

For GSK-3β, synapsin I and BAG-1 gene expression analysis, total hippocampal RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA

synthesis was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Semiquantitative PCR reactions were performed as previously described (Wong *et al.,* 1994). Briefly, each PCR cycle included the following steps: 94 °C for 30 s, 57 °C for 45 s and 72 °C for 60 s. A sequential series of PCR reactions using each primer pair was initially run to determine optimal annealing temperature and cycle number to ensure amplification within the exponential phase of the amplification curve for both the gene under study and the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT). The expression level of the reference gene (HPRT) was used as an internal standard, to which other PCR amplification products were normalized. HPRT was chosen as a reference gene since no variation in its expression was observed between groups. The oligonucleotide primers for GSK-3β, synapsin I, BAG-1 and HPRT were synthesized using the Primer3 software on the basis of the following GenBank sequences: NM_032080 (GSK-3β); NM_019133 (synapsin I); NM_007860 (BAG-1) and NM_012583 (HPRT). The sequences of oligonucleotide primers were: GSK-3β sense, TTGGAAATGGGTCATTTGGT; GSK-3β anti-sense, TCACAGGGAGTGTCT GCTTG; synapsin I sense, CAGGGTCAAGGCCGCCAGTG; synapsin I anti-sense, CACATCC TGGCTGGGTTTCTG; BAG-1 sense, ATGGAAACACCCTTGTCAGC; BAG-1 anti-sense, AAAACCCTGCTGGATGTCAG; HPRT sense GCAGACTTT GCTTTCCTTGG; HPRT anti-sense TCCACTTTCGCTGATGACAC. The values presented are the average of 3 PCR runs for each sample.

Immunocytochemical detection of mitosis, apoptosis and differentiation

Proliferation was assessed by immunocytochemistry (ICC) for BrdU incorporation (daily injections for 3 days) (Fig. 1.1) and confirmed by Ki-67 (an endogenous restricted marker) (Fig. 1.2). As previously described (Silva *et al.,* 2006) BrdU positive cells were detected by ICC (mouse monoclonal anti-BrdU, 1:50, Dako, Glostrup/DK) in every 8th section. For Ki-67, ICC

representative sections of dorsal and ventral hippocampi were deparaffinized, immersed in TBS-T (0.1%) for 5 minutes and microwaved while immersed in citrate buffer (0.1 M) for 15 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 in TBS (10 minutes) and non-specific staining was blocked with 4% bovine serum albumin (BSA) in TBS (30 minutes). Subsequently, sections were incubated for 1.5 hours with a mouse monoclonal anti-Ki-67 antibody (1:200, Novocastra, Newcastle Upon Tyne, UK). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and diaminobenzidine (DAB: 0.025% and 0.5% H_2O_2 in Tris-HCl 0.05M, pH 7.2). Specimens were lightly counterstained with hematoxylin.

Apoptosis was estimated using morphological criteria and TUNEL histochemistry (Fig. 1.3) (TdT, BI Fermentas, Hanover/MD); dUTP-Biotin, Roche, Basel/Switzerland) in every 8th section, as previously described (Silva *et al.,* 2006). In addition cell proliferation and death in the subventricular zone (SVZ) was estimated by measuring BrdU, Ki-67 and TUNEL positive cells in 16 sections evenly distributed along the hemispheric anterior-posterior axis.

The phenotype of newly-acquired cells was assessed by double staining of proliferation markers (BrdU and Ki-67) with differentiation markers (DCX for immature neurons, NeuN for mature neurons and GFAP for astrocytes). Using the above described protocol, sections representative of the dorsal and ventral hippocampi were double stained for Ki-67 (mouse anti-Ki-67; 1:200, Novocastra, Newcastle Upon Tyne, UK) and DCX (rabbit anti-DCX polyclonal antibody, 1:500, Abcam, Cambrige/UK) (Fig. 1.4). The following secondary antibodies were used: anti-mouse Alexa Fluor-568 (Ki-67), anti-rabbit Alexa Fluor-488 (DCX) (all 1:200, Molecular Probes, Eugene/OR). A set of sections representative of the dorsal and ventral hippocampi was sequentially double stained for BrdU (rat anti-BrdU monoclonal antibody, 1:50, Abcam, Cambrige/UK) and NeuN (mouse anti-NeuN, 1:100, Chemicon, Temecula/CA) (Fig. 1.5), while another set was sequentially stained for BrdU (mouse anti-BrdU monoclonal antibody, 1:50,

Dako, Glostrup/DK) and GFAP (rabbit anti-GFAP, 1:200, Dako, Glostrup/DK) (Fig. 1.6). The following secondary antibodies were used: anti-rat Alexa Fluor-488 (BrdU), anti-mouse Alexa Fluor-568 (NeuN), anti-mouse Alexa Fluor-568 (BrdU), anti-rabbit Alexa Fluor-488 (GFAP) (all antibodies were used at a concentration of 1:200, Molecular Probes, Eugene/OR).

For each animal, 50-100 BrdU or Ki-67-positive cells within the dentate gyrus were analyzed after double-staining with neuronal or glial markers, using an Olympus FV1000 confocal microscope.

Detection of hippocampal GSK-3β and synapsin-I immunoreactivity

Glycogen synthase kinase-3β (GSK-3β) (Fig. 2.1) and synapsin-I (Fig. 2.2) were detected by immunocytochemistry in 16 sections per brain; sections were evenly distributed along the hippocampal anterior-posterior axis. Briefly, sections were deparaffinized and microwaved while immersed in citrate buffer (0.1 M) for 20 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 in TBS (10 minutes) and non-specific staining was blocked with 4% bovine serum albumin (BSA) in TBS (30 minutes). Subsequently, sections were incubated overnight with a mouse monoclonal anti-GSK-3β (1:100, Lab Vision, Fremont, CA) or mouse anti-synapsin-I (1:100, Sigma, St Louis, MO). Antigen visualization was carried out using an universal detection system (BioGenex, San Ramon, CA) and diaminobenzidine (DAB: 0.025% and 0.5% H_2O_2 in Tris-HCl 0.05M, pH 7.2). Specimens were lightly counterstained with hematoxylin.

Quantification procedures

As previously described (Silva *et al.,* 2006), proliferation and apoptotic densities were estimated in the different subdivisions of the hippocampus using the *StereoInvestigator* software (MicroBrightField, Williston/VT) and the optical fractionator method with a sampling size area of

350 x 350 µm for the molecular layer and the hilus and 100 x 150 µm for both the granule cell layer and subgranular zone. The area of the counting frame was 30 x 30 µm. For topographic analysis, the hippocampal dentate gyrus was divided into its main subfields: molecular layer (ML), granular cell layer (GCL), sugranular zone (SGZ) and hilus (Hi).

To determine GSK-3β and synapsin I density, the *Alphalmager* program (Alphalnnotech, San Leandro, CA) was used. Densities were calculated by subtracting the optical density of the area of interest by the optical density of an adjacent area, thus eliminating background effects.

Statistics

The results are expressed as group means ± standard error of the mean (SEM). Coefficients of error (CE) were calculated accordingly to Gundersen & Jensen (1987). Statistical analysis was preformed using SPSS 14.0 software (SPSS Inc., Chicago/IL). The effects of Cort, Dex, ADX and lithium *per se* were analysed by independent-sample t-test, while the actions of lithium when co-administrated (Cort+Li, Dex+Li and ADX+Li) were assessed through two-way analysis of variance (ANOVA). Statistical significance was accepted when p was ≤ 0.05.

Results

Biometric, hormonal and behavioural markers

Several biometric parameters were influenced by the experimental procedures as detailed in Table 1.

The forced-swimming test was used to monitor depressive-like behaviour. Both Cort, Dex and ADX rats displayed increased immobility time ($p \leq 0.008$); in addition, latency to immobility was decreased only by Dex and ADX treatments ($p \leq 0.006$) (Fig. 3.1 – 3.3). In summary, manipulations of corticosteroid milieu induced a depressive-like behaviour. Two-way ANOVA revealed that concomitant administration of lithium prevented these Cort, Dex and ADX behavioral effects ($p \leq 0.02$), with the exception of ADX-induced increase in immobility time (Fig. 3.1 - 3.3). However, it was also possible to see an increased immobility time in animals treated only with Li ($p < 0.008$) (Fig. 3.4).

The effects of manipulations in the corticosteroid milieu in hippocampal cell final balance are prevented by lithium

Corticosterone (Cort) and dexamethasone (Dex) treatment led to a decrease of both markers (BrdU and Ki-67) of proliferation ($p < 0.04$) and an increase of apoptosis (TUNEL) ($p < 0.03$) in hippocampal dentate gyrus (Fig. 4.1 to 4.6). These data suggests that high GR activation results in significant decrease of hippocampal dentate gyrus cell balance ($p \leq 0.03$; Fig. 4.7 and 4.8). Two-way ANOVA showed that lithium prevented Cort and Dex effects in hippocampal proliferation and apoptosis ($p \leq 0.02$), apart from the DEX-induced apoptosis in the hilus (Fig. 4.6).

Removal of the adrenal glands (ADX) induced an increment in the density of mitotic (BrdU and Ki-67) and apoptotic (TUNEL) cells in dentate gyrus main areas ($p \leq 0.008$) (Fig. 5.1 to 5.3). As

indicated by two-way ANOVA lithium co-treatment (ADX+Li) led to even higher proliferating indexes ($p \leq 0.03$) (Fig. 5.1 and 5.2). Moreover, lithium was able to diminish the ADX-induced effects in apoptosis in all areas of the dentate gyrus ($p \leq 0.03$), except in the Hi (Fig. 5.3); importantly, this reduction in apoptosis was not only marginal, as there was still a significant difference of ADX+Li rats when compared to controls. The combined effects of ADX in proliferation and apoptosis resulted in a reduction of the final cell balance ($p \leq 0.002$); interestingly, this was precluded by concomitant lithium administration as a result of the increased proliferation ($p \leq 0.0005$) (Fig. 5.4).

As shown in Figure 6 lithium administration to stress-free animals led to increased proliferation (BrdU and Ki-67) and apoptosis (TUNEL) ($p \leq 0.05$) (Fig. 6.1 to 6.3). As the increases in proliferation outweighed those of proliferation, the final cell balance in all areas of the hippocampal dentate gyrus was higher than that of controls ($p < 0.05$), with the exception of the GCL ($p \leq 0.001$) (Fig. 6.4).

Besides the effects in proliferation, alterations in the corticosteroid milieu affected the cell differentiation process. All corticosteroid manipulations decreased the neuronal differentiation (BrdU+NeuN and Ki-67+ DCX) in favour of a glial phenotype (BrdU+GFAP) ($p \leq 0.008$); these effects were blocked by lithium co-administration ($p \leq 0.001$) (Table 2). Furthermore, lithium alone increased both neuronal and glial differentiation ($p \leq 0.0005$) (Table 2).

Impact of corticosteroid milieu imbalances in hippocampal GSK3β, synapsin-1 and BAG-1: molecular targets for lithium actions

The analysis of GSK-3β mRNA and protein levels revealed an increase after Dex treatment and a decrease in ADX animals ($p \leq 0.04$) (Fig. 7.1 to 7.6). While the effect of Dex was blocked by lithium co-administration, the decrease in ADX was further accentuated by lithium ($p \leq 0.03$) (Fig. 7.3 to 7.6). Interestingly, no significant effects on GSK-3β expression were observed with Cort treatment (Fig. 7.1 and 7.2).

The ability of lithium to prevent the effects of corticotherapy on GSK-3β suggests the involvement of this pathway in determining cell-fate. Corticosteroids are modulators of pro- and anti-apoptotic molecules (for review see Sousa and Almeida 2002). Interestingly, Li also targeted the apoptotic pathway, as shown by its influence on the expression of the anti- apoptotic molecule BAG-1 (Zhou et al., 2005). Here we analysed BAG-1 gene expression and it was possible to observe that the hippocampal mRNA levels of BAG-1 were decreased in Dex and ADX ($p \leq 0.001$) (Fig. 8.1 to 8.3); both effects were prevented by lithium co-administration ($p \leq 0.03$) (Fig. 8.2 and 8.3). Consistently with previous reports (Zhou et al., 2005) lithium *per se* also increased BAG-1 expression ($p \leq 0.03$) (Fig. 8.4).

As GSK-3β is also implicated in impaired synaptic plasticity and cognition (see Manji 2001), we monitored the expression of a presynaptic marker, synapsin-I, a known target of GSK3β (Hall et al., 2000). This analysis revealed that Dex and ADX animals displayed decreased mRNA and protein levels of synapsin-I ($p \leq 0.02$) (Fig. 9.1 to 9.6). Concomitant administration of lithium preclude the Dex-induced effects in the protein levels of synapsin-I at the stratum radiatum of

CA3 but aggravated the ADX actions on synapsin-I mRNA and protein levels in the same region ($p \leq 0.05$) (Fig. 9.3 and 9.6). Corticosterone treatment did not significantly influence this synaptic protein (Fig. 9.1 and 9.2).

Discussion

Recently we showed an association between depressive-like behaviour induced by stress and decrease hippocampal granule cell balance (Silva et al., 2007). This association was paralleled by an increase of GSK-3β and a decrease of BAG-1 (an anti-apoptotic protein) and of the pre-synaptic protein synapsin-1; importantly, lithium co-administration, through regulation of GSK-3β, blocked these deleterious actions (Silva et al., 2007). In the present study, we went further on this analysis by discriminating the role of each corticosteroid receptors in these effects.

High GR activation, as a consequence of either corticosterone or dexamethasone treatment, triggered a depressive-like behaviour. However, because dexamethasone-treated animals displayed changes in latency to immobility and immobility times, while corticosterone only influenced immobility time, one could argue that the behavioral impact of exclusive GR activation is more severe than that resulting from the use of a mixed ligand. Interestingly, adrenalectomized animals also displayed signs of increased learned helplessness, showing that unoccupancy of MR and GR also triggers depressive-like behavior. Taken together, these results, which are in accordance with previous works (Holsboer et al., 2000), seem to indicate that the imbalances in corticosteroid milieu are, in fact, relevant for depression in rodents and that both corticosteroid receptors operate in this event.

The possible involvement of neurogenesis in the etiology of depression (Malberg et al., 2000; Santarelli 2003), prompted us to assess the role of MRs and GRs in the alterations induced by hypercortisolemia in hippocampal neurogenesis and apoptosis (Silva et al., 2007). High GR activation induced a reduction in dentate granule cell turnover, as a consequence of decreased proliferation and increased apoptosis; interestingly, these effects were again striker after Dex than

with Cort administration. Although adrenalectomy also reduced the granule cell turnover, consistently with previous reports (Sloviter et al., 1989; Greiner et al., 2001) this effect was largely due to the remarkable enhancement of apoptosis triggered by this condition. These results add support to the perspective that impaired hippocampal cell survival contributes to depression (Lucassen et al., 2004), but emphasize that the balance of cell gains and losses gives a better insight than the proliferation or apoptosis indexes alone.

Consistent with the recent evidence that stress modulates GSK-3β expression and activity (Silva et al 2007), we herein confirmed that GR are critical in mediating this effect (Sotiroupolos et al 2006). In fact, exclusive GR activation induced an increased in GSK-3β, while GR unoccupancy after ADX resulted in decreased levels of this kynase. Interestingly, the impact of high GR and MR activation in the expression of GSK-3β, was not evident, which suggests that MR activation might play an inhibitory role in its expression. Important for the context of this study is the link between GSK-3β activity, corticosteroid receptors and depressive-like behavior. Even thought caution is required when extrapolating for this association, it is pertinent to remember that specific (AR-A014418) and unspecific (e.g. lithium) inhibitors of GSK-3β were recently proved to have antidepressant properties (Stambolic et al., 1996; Jope, 2003; Gould et al., 2004, Silva et al., 2007). Moreover, in the present study, lithium proved to be efficient in blocking the behavioral and cell fate actions resulting from corticosteroid imbalances in parallel with a restoration of the levels of hippocampal GSK-3β expression.

One of the most interesting downstream targets of GSK-3β is BAG-1. Interestingly, it seems that either directly or indirectly, GSK-3β modulators (e.g. lithium) can recruit proteins belonging to the Bcl-2 family of anti-apoptotic molecules, as BAG-1 (Zhou et al., 2005). The protein BAG-1 besides

increasing the anti-apoptotic capacities of Bcl-2 (Takayama et al 1995; Manji et al., 2000), also influences the glucocorticoid receptor (GR) activity due to its co-chaperone mechanisms (Schneikert et al 1999). Therefore, in accordance with previous reports of increase apoptosis after exclusive GR activation (Hassan et al., 1996, Almeida et al., 2000) or MR unoccupancy (Sloviter et al., 1989, Sousa and Almeida, 2002) and with our present TUNEL staining, we observed a decreased hippocampal BAG-1 expression after Dex administration or after ADX. In contrast, lithium co-treatment was able to prevent the Dex-induced inhibitory effects on this anti-apoptotic protein.

Inhibition of GSK3β is also known to increase levels and clustering of the pre-synaptic marker, synapsin-I (Hall et al 2002), revealing an ability to modulate synaptic plasticity. The observation that Dex treatment reduced hippocampal synapsin-I expression is indicative of impaired hippocampal synaptic activity, which fits previous observations showing that the synaptic structure (Sousa et al., 2000) and function (Kim and Diamond, 2002) is impaired after sustained activation of GR. Despite a trend for a reduction in the expression of this synaptic protein, Cort treatment failed to produce a significant effect; this fact supports the neuroprotective role of MR, which, in accordance with other works (Krugers et al., 2007), is further reinforced by the reduction observed in ADX animals.

As depression is one of the major contributors to the global illness burden, it is important to better understand its underlying mechanisms. In the present study, we confirmed an association between corticosteroid imbalances, depressive-like behavior and hippocampal granule cell balance; we have further determined the role of MR and GR in this phenomenon. Subsequently, by showing the involvement of GSK-3β, and some of its downstream targets, in these events we

established a novel pathway through which lithium, a mood stabilizer with antidepressant properties, prevents the appearance of the behavioral and cellular effects triggered by corticosteroids imbalances.

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Tables

Table 1. Biometric and endocrine parameters

	Cont	Cort	Cort+Li	Dex	Dex+Li	ADX	ADX+Li	Li
Δ body weight (g)	131 (0.9)	73* (1.8)	69* (1.5)	48* (0.6)	39** (0.9)	113* (0.1)	112* (2.1)	120* (1.4)
Thymus weight (g)	0.56 (0.003)	0.0* (0.0)	0.0 (0.0)	0.0* (0.0)	0.0 (0.0)	0.58* (0.007)	0.62** (0.008)	0.57 (0.005)
Adrenal weight (g)	0.042 (0.002)	0.017* (0.0007)	0.017* (0.0013)	0.016* (0.0002)	0.018** (0.004)	0.0* (0.0)	0.0* (0.0)	0.048* (0.0003)
Plasmatic levels of Corticosterone (ng/mL)	68 (20)	315* (26)	316 (27)	5* (3)	9 (2)	0,6* (0.1)	0,4 (0.1)	90 (15)

Groups: control (Cont), corticosterone (Cort), corticosterone + lithium (Cort+Li), dexamethasone (Dex), dexamethasone + lithium (Dex+Li), adrenalectomy (ADX) adrenalectomy + lithium (ADX+Li) and lithium (Li). Values are expressed as means (± SEM). * significant to Cont group; ** significant to the respective lithium-free group.

Table 2. Percentage of newly-acquired cells that co-expressed neuronal or glial markers

	Cont	Cort	Cort+Li	Dex	Dex+Li	ADX	ADX+Li	Li
BrdU+NeuN	48.2 (1.7)	25.4* (0.8)	32.9** (2.9)	24.5* (2.8)	37.5** (1.3)	25.1* (3.6)	40.6** (1.5)	54.7* (1.2)
Ki-67+DCX	40 (0.7)	22.9* (1.0)	31.9** (1.2)	20.0* (2.3)	28.7** (2.1)	28.9* (0.6)	38.5** (1.4)	50.6* (1.7)
BrdU+GFAP	3.9 (0.6)	18.9* (2.2)	21.4** (2.2)	13.5* (0.4)	23.2*,** (1.5)	13.4* (0.7)	15.0** (0.7)	16.7* (1.7)

Groups: control (Cont), corticosterone (Cort), corticosterone + lithium (Cort+Li), dexamethasone (Dex), dexamethasone + lithium (Dex+Li), adrenalectomy (ADX) adrenalectomy + lithium (ADX+Li) and lithium (Li). Values are expressed as means (± SEM). * significant to Cont group; ** significant to the respective lithium-free group.

Figures

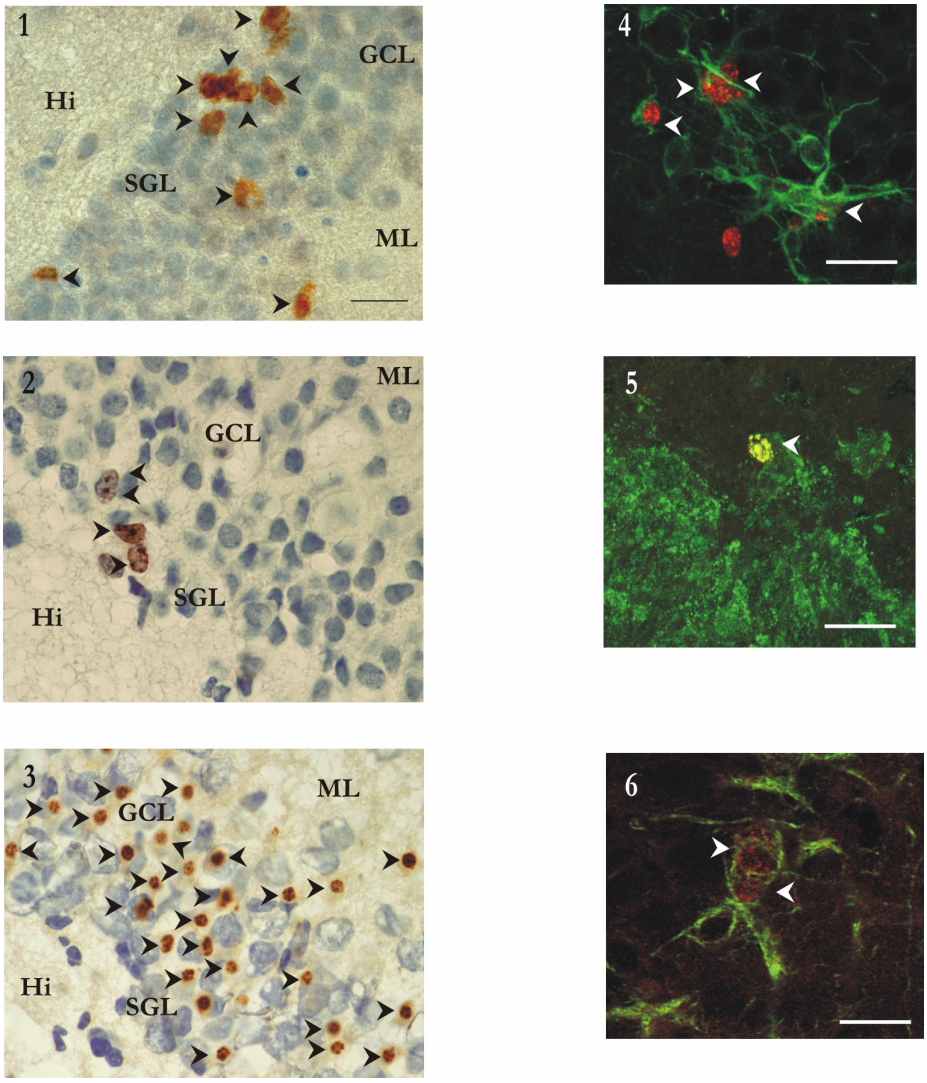

Fig.1 Photomicrograph illustrating immunostaining for BrdU **(1.1)**, Ki-67 **(1.2)** and TUNEL positive cells **(1.3)** (arrowheads) in the hippocampal dentate gyrus (Scale bar: 80 μm). Molecular layer (ML), granule cell layer (GCL), subgranular zone (SGZ) and hilus (Hi).

Confocal photomicrographs illustrating colocalization of Ki-67+DCX, BrdU+NeuN and BrdU+GFAP. **(1.4)** Proliferating cells labeled with Ki-67 (red) and DCX (green) (scale bar: 10 μm); **(1.5)** proliferating cells labelled with BrdU (green) and neuronal cells labeled with NeuN

(red) (scale bar: 30 μm). **(1.6)** Proliferating cells labeled with BrdU (red) and glial cells labeled with GFAP (green) (scale bar: 10 μm). Arrowheads indicate double-labeling.

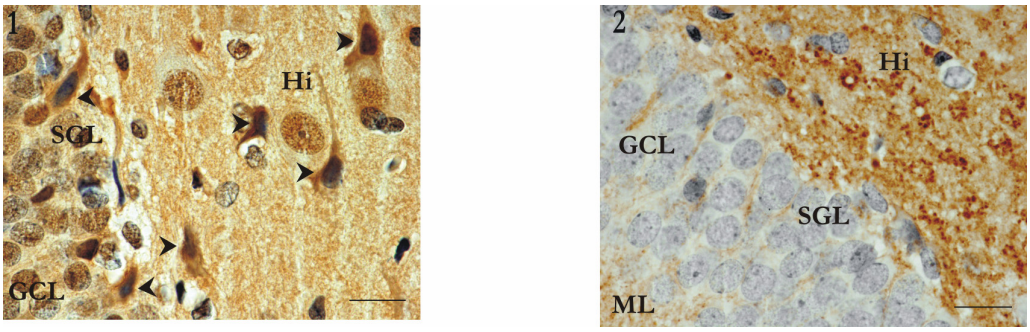

Fig. 2. Photomicrographs illustrating hippocampal dentate gyrus immunostained for GSK-3β **(2.1)** and synapsin-1 **(2.2)**. Scale bars: 50 μm.

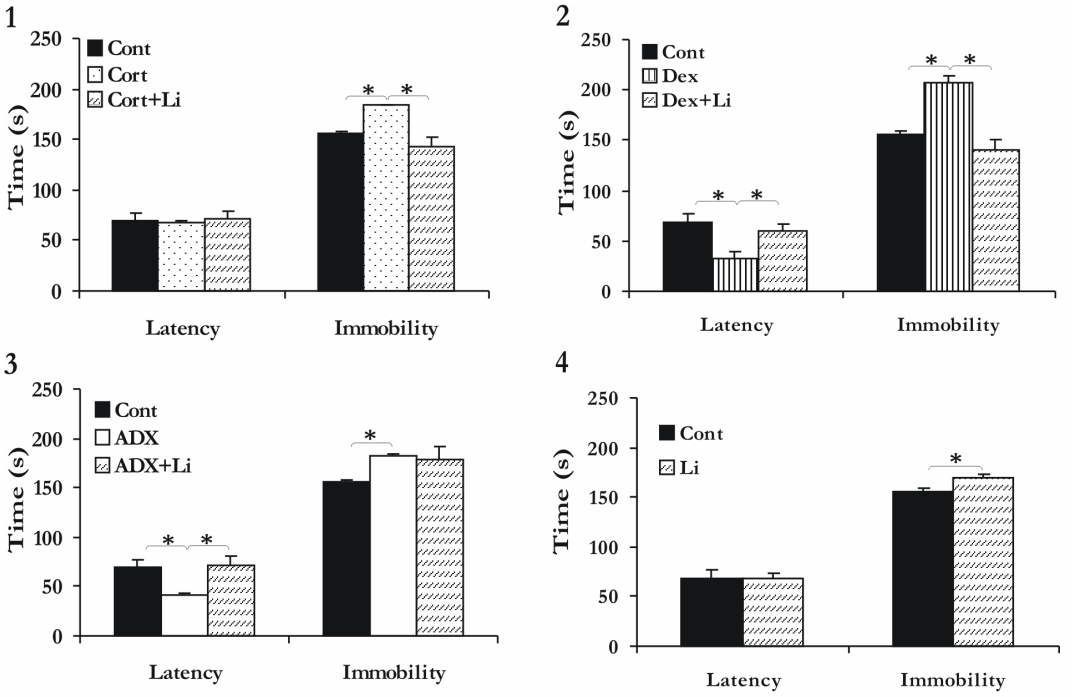

Fig. 3. Graphic representation of the latency and immobility times in the FST of control (Cont), corticosterone (Cort), corticosterone + lithium (CMS+Li), dexamethasone (Dex), dexamethasone + lithium (Dex+Li), adrenalectomy (ADX) and adrenalectomy + lithium (ADX+Li) animals. It was possible to observe an increase in the immobility of Cort, Dex and ADX **(3.1** to **3.3)** and a decrease in the latency times of Dex and ADX animals when compared with controls **(3.2** and **3.3)**. Co-administration of lithium prevented the increase in immobility of Cort and Dex **(3.1** and **3.2)** and the decrease in the latency time of Dex and ADX animals **(3.2** and **3.3).** Lithium *per se* did not influence latency but lead to an increase in the immobility time **(3.4)**.

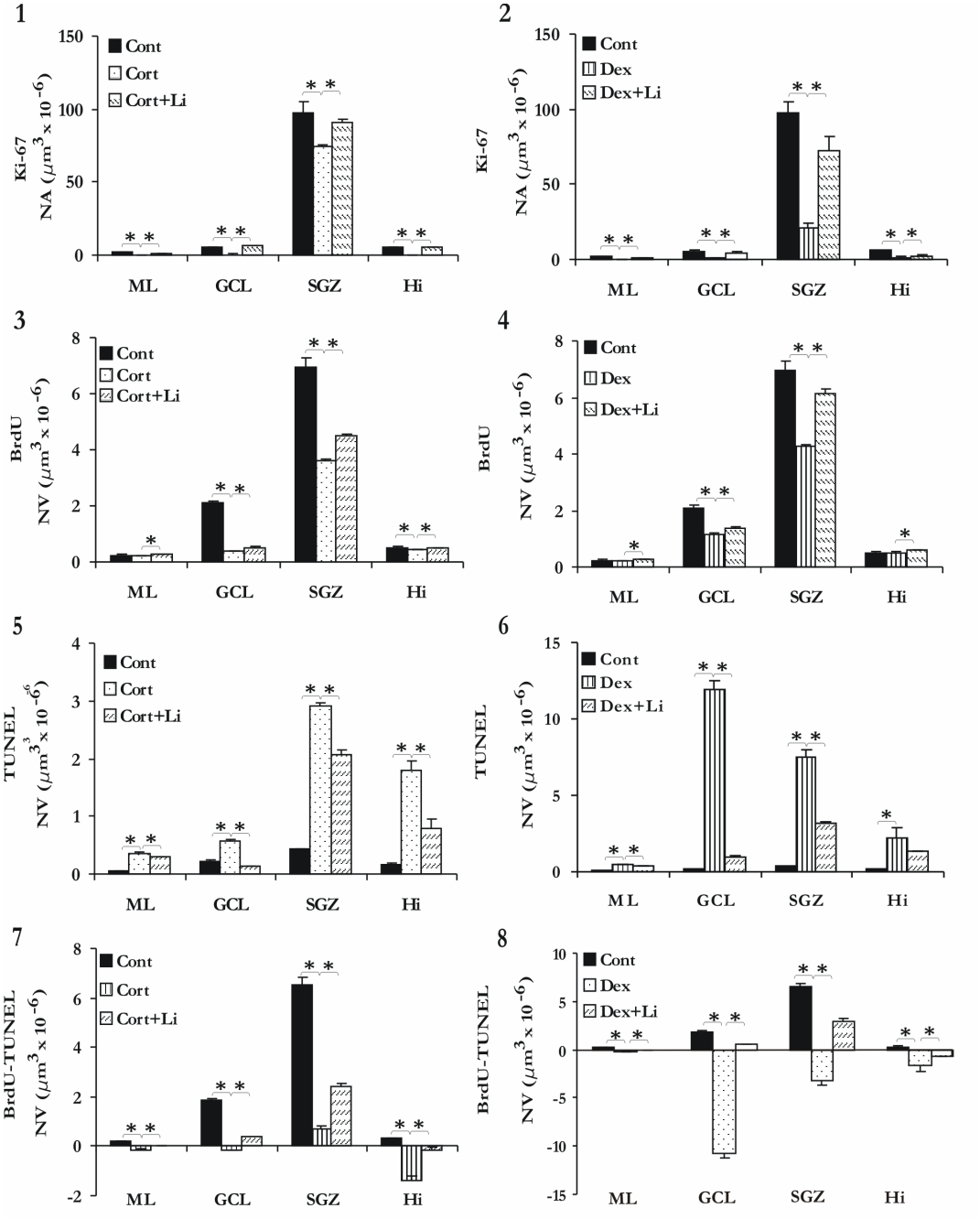

Fig. 4. Graphic representations of the comparison between control (Cont), corticosterone (Cort), corticosterone + lithium (CMS+Li), dexamethasone (Dex) and dexamethasone + lithium (Dex+Li), for proliferation, apoptosis and final cell balance in the hippocampal dentate gyrus (DG). It was possible to see a decrease in the indexes of proliferation (Ki-67 and BrdU) in the hippocampal dentate gyrus of Cort and Dex animals, with the exception of the ML of Cort and the ML and Hi of

Dex rats **(4.1** to **4.4).** Co-administration of lithium prevented the Cort- and Dex-induced reduction in proliferation (Ki-67 and BrdU) **(4.1** to **4.4)**. When apoptosis (TUNEL) was analysed, it revealed an increase of apoptotic indexes both with Cort and Dex in all dentate gyrus areas, which was partially precluded with lithium administration **(4.5** and **4.6)**. The exception to these reversions was the Hi of Dex animals **(4.6)**. The BrdU-TUNEL balance showed a decrease to negative values with Cort and Dex administrations, excepting the SGZ of the Cort rats were the balance was lower than controls but not negative **(4.7** and **4.8)**. Administration of lithium partially prevented the Cort and Dex effects **(4.7** and **4.8)**.

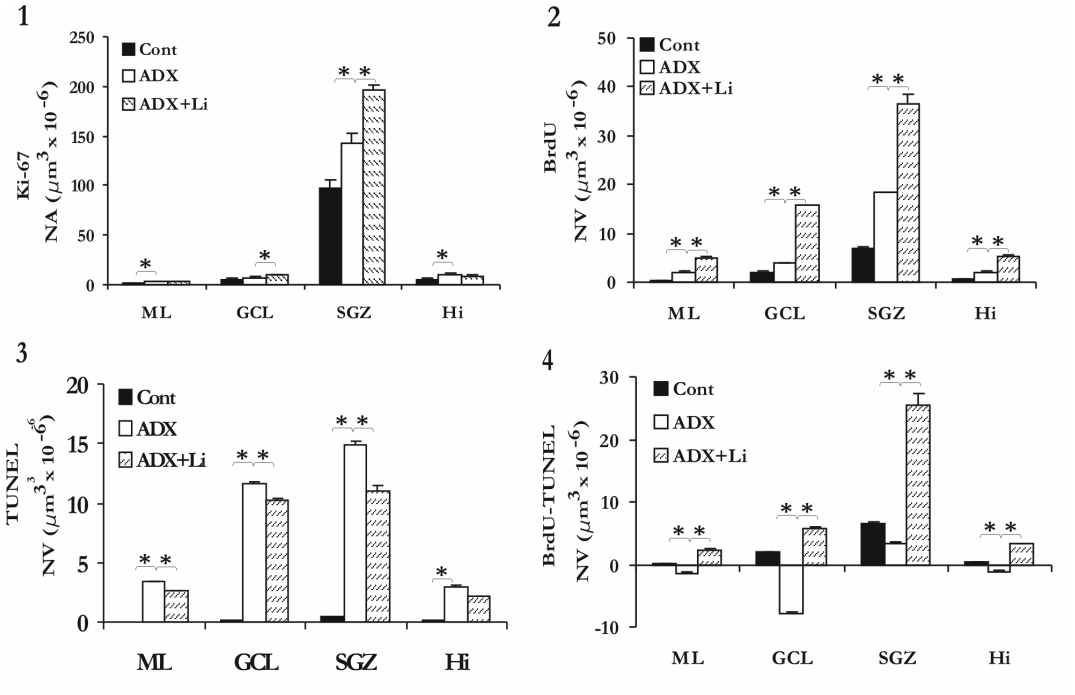

Fig. 5. Graphic representations of the comparison between control (Cont), adrenalectomyzed (ADX), adrenalectomyzed + lithium (ADX+Li) for proliferation, apoptosis and final cell balance in the hippocampal dentate gyrus. Adrenalectomy increased the cell proliferation (Ki-67 and BrdU) and death in all dentate gyrus areas **(Fig. 5.1** to **5.3)**. Concomitant injections of lithium to adrenalectomy led to even higher mitotic indexes but decrease apoptotic density, excepting in the and Hi **(Fig. 5.1** to **5.3)**. Although adrenalectomy increased both parameters, the cell turnover was negative in all dentate gyrus areas, with the exception of SGZ, where it had positive but even though lower levels than control animals **(Fig. 5.4)**. Lithium administration precluded the ADX effects in the final cell balance **(Fig. 5.4)**.

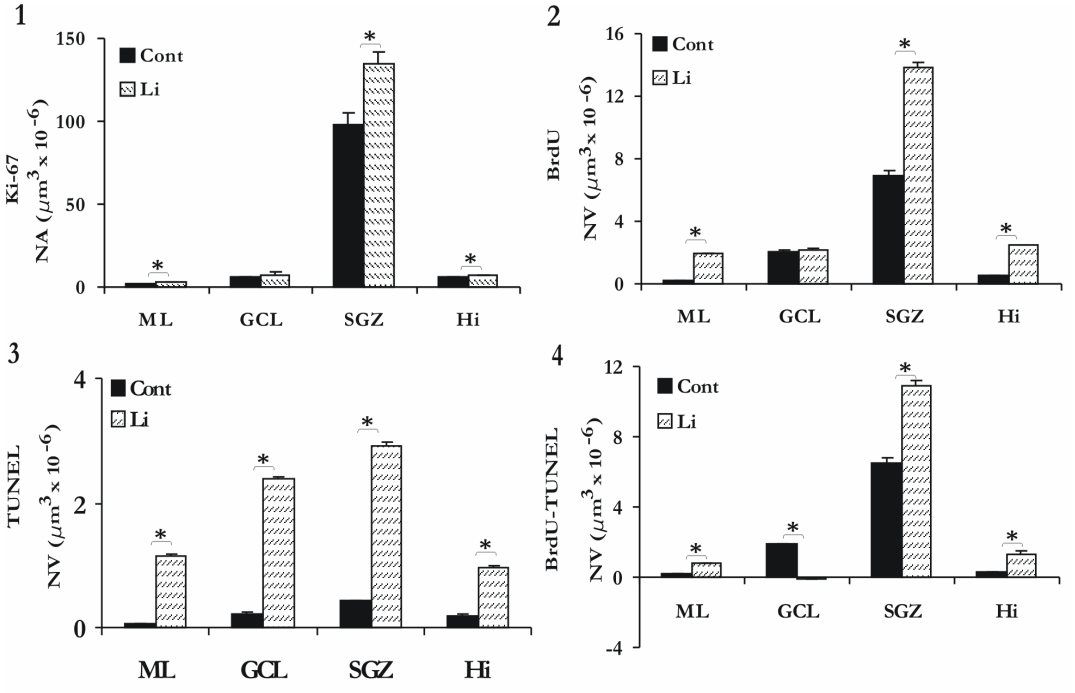

Fig. 6. Graphic representations of the comparison between control (Cont), and lithium (Li) for proliferation, apoptosis and final cell balance in the hippocampal dentate gyrus. Administration of lithium in a non insult context led to a parallel increase of proliferation (Ki-67 and BrdU) and apoptotic indexes **(Fig. 6.1** to **6.3)**. Lithium ambiguous actions are also present in final cell balance, whereas in the GCL it seems to be deleterious in the ML, SGZ and Hi it increased the final cell balance **(6.4)**.

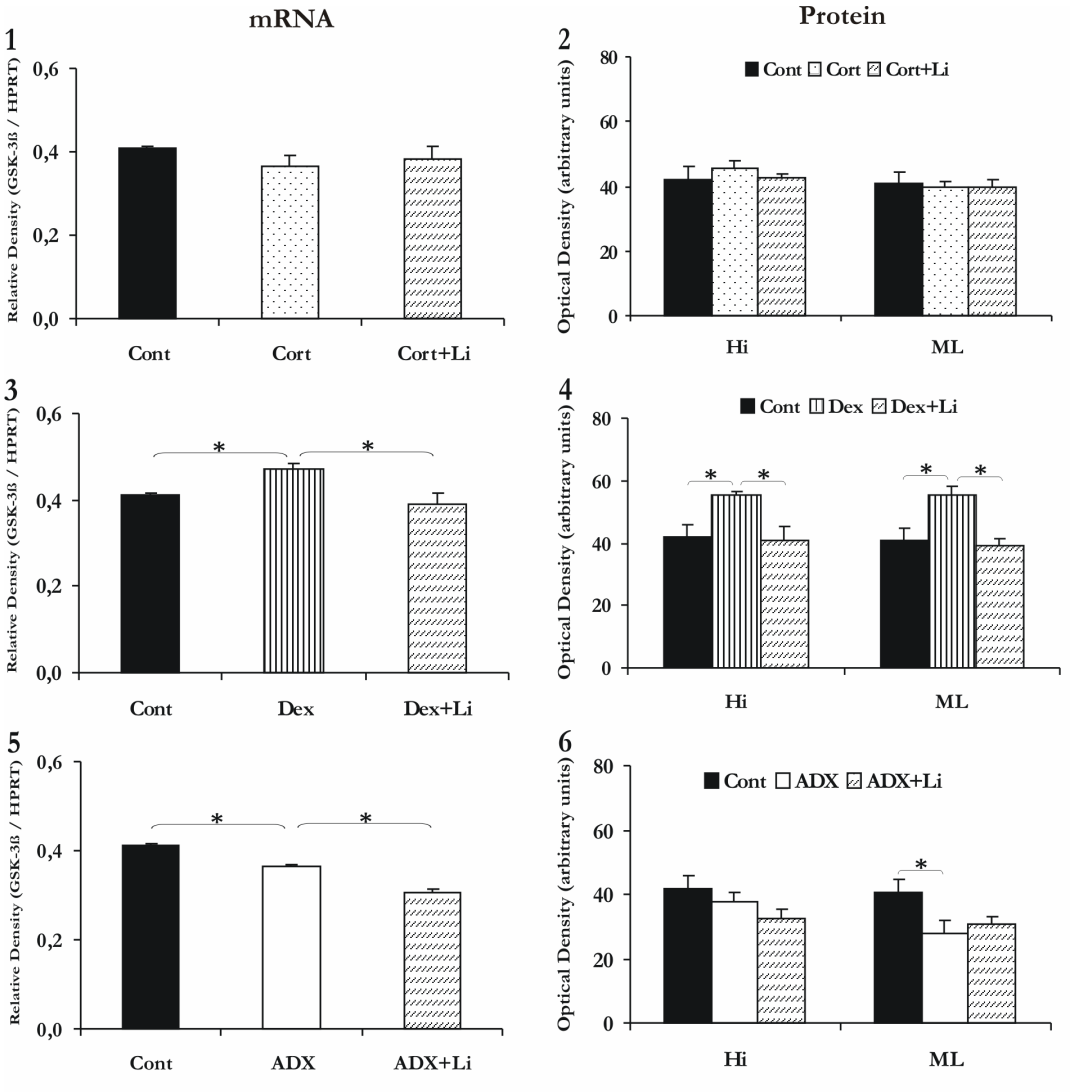

Fig. 7. Graphic representations of the GSK-3β comparisons between control (Cont), corticosterone (Cort), corticosterone + lithium (CMS+Li), dexamethasone (Dex), dexamethasone + lithium (Dex+Li), adrenalectomyzed (ADX), adrenalectomyzed + lithium (ADX+Li) and lithium (Li). Analysis of GSK-3β hippocampal mRNA levels and of the density of GSK-3β expressing cells in dentate gyrus molecular layer (ML) and hilus (HI) showed that although corticosterone (Cort) did not induce a variation **(Fig .7.1** and **7.2)** dexamethasone increased both parameters **(Fig 7.3** and **7.4)**. Lithium co-administration with Dex (Dex+Li) prevented Dex effects **(Fig 7.3** and **7.4)**. Adrenalectomy (ADX) led to a decrease in GSK-3β mRNA and protein (ML) levels **(Fig. 7.5** and

7.6). Animals which were adrenalectomyzed and concomitantly received injections of lithium (ADX+Li) presented an even lower expression of GSK-3β than ADX group **(Fig. 7.5)**.

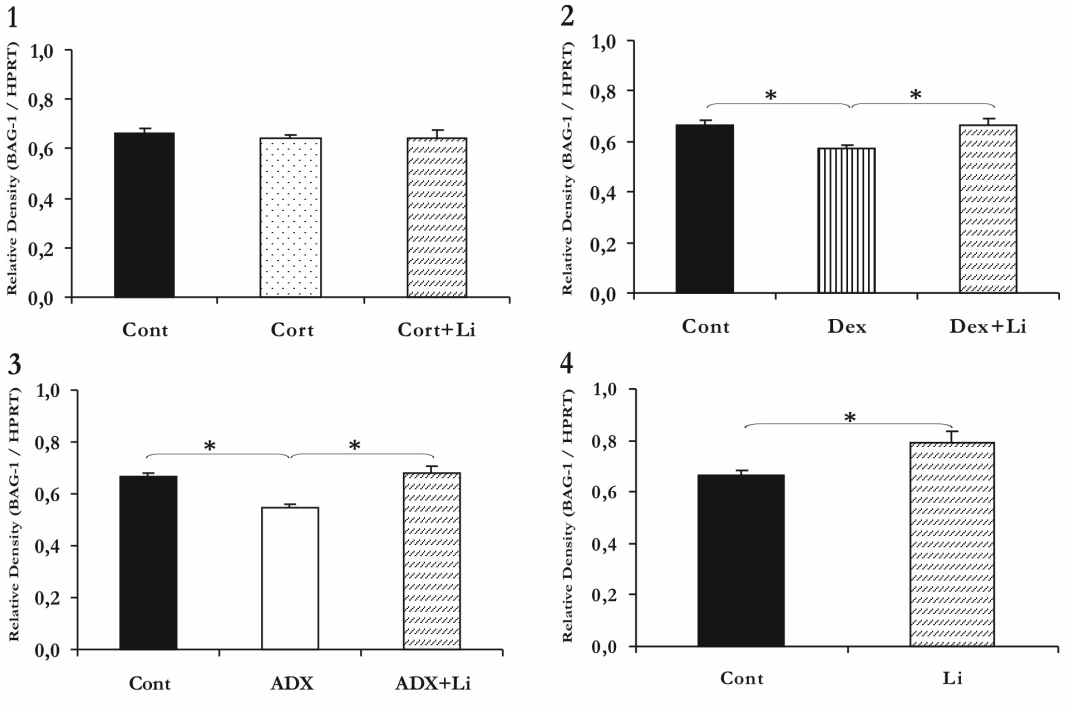

Fig. 8. Graphic representations of the comparisons of Bcl-2-associated athanogene protein-1 (BAG-1) gene expression, between control (Cont), corticosterone (Cort), corticosterone + lithium (CMS+Li), dexamethasone (Dex), dexamethasone + lithium (Dex+Li), adrenalectomyzed (ADX), adrenalectomyzed + lithium (ADX+Li) and lithium (Li). Despite the no induction of variation by corticosterone (Cort), dexamethasone (Dex) and adrenalectomy (ADX) reduced BAG-1 mRNA levels **(8.1** to **8.3)**. Lithium co-administration (Dex+Li and ADX+Li) precluded Dex and ADX actions **(8.2** to **8.3)**. Administration of lithium *per se* also increased the mRNA levels of BAG-1 **(8.4)**.

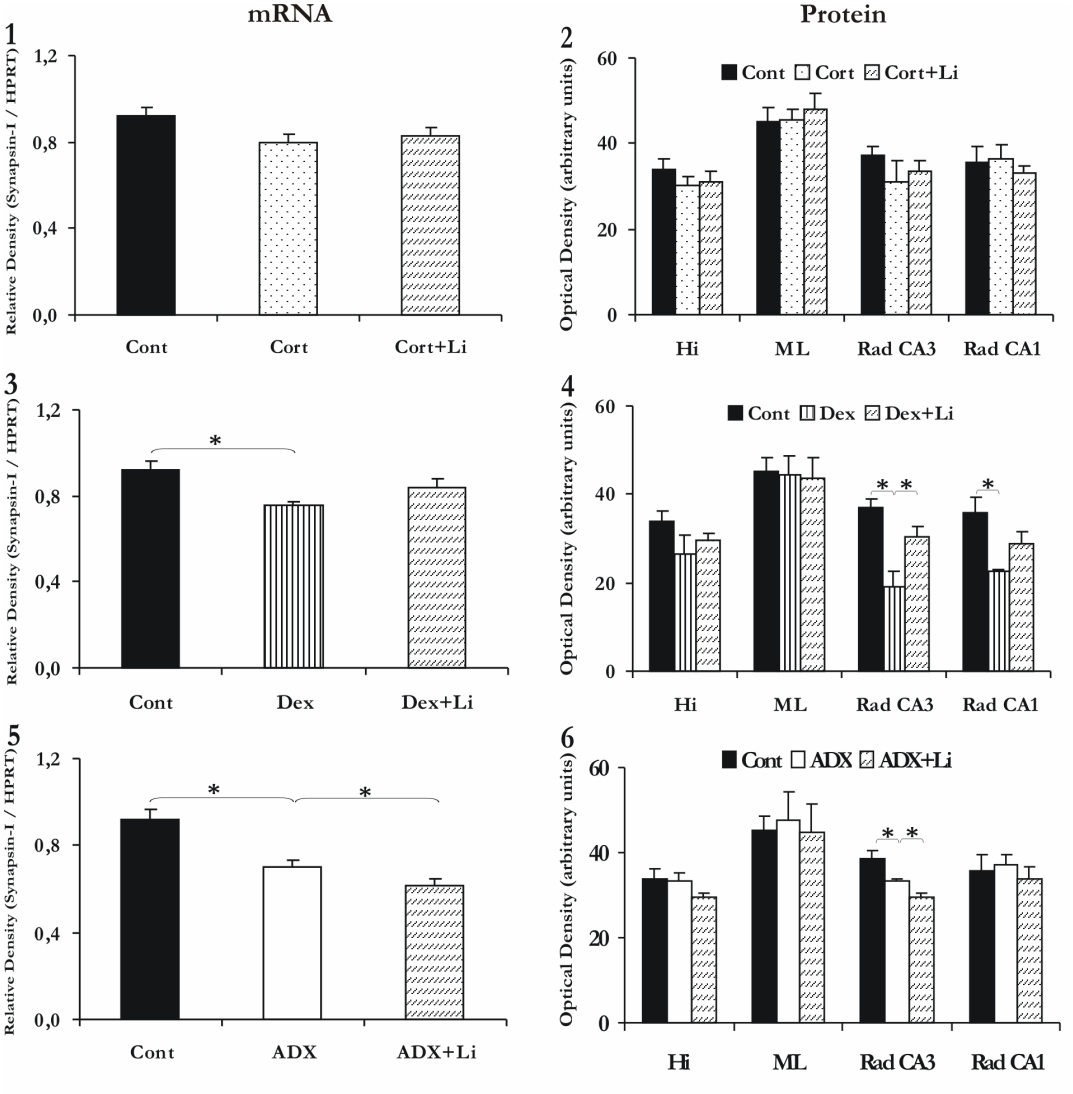

Fig. 9. Graphic representations of the comparisons of synapsin-I hippocampal mRNA levels and density of synapsin-I expressing cells in the molecular layer (ML) and hilus (HI) of the dentate gyrus and in radiatum layer of CA3 and CA1 fields, between control (Cont), corticosterone (Cort), corticosterone + lithium (CMS+Li), dexamethasone (Dex), dexamethasone + lithium (Dex+Li) , adrenalectomyzed (ADX), adrenalectomyzed + lithium (ADX+Li) and lithium (Li). Dexamethasone (Dex) but not corticoisterone (Cort) animals displayed lower levels of synapsin-I mRNA and protein **(9.1** to **9.4)**. Although lithium co-administration did not have effect when co-administrated with Cort (Cort+Li), it prevented (Dex+Li) Dex-induced decrease of synapsin-I in

Rad CA3 **(9.1** to **9.4)**. Adrenalectomy (ADX) reduced protein and mRNA levels of synapsin-I **(9.5** to **9.6)**, with lithium co-administration (ADX+Li) not altering both parameters **(9.5** to **9.6)**.

Chapter 2.4

Silva R, Martins L, Longatto-Filho A, Almeida OFX and Sousa N

Vascular endothelium growth factor as a potential mediator of the neurogenic actions of lithium

(Submitted to Neuroscience Letters)

Vascular endothelium growth factor as a potential mediator of the neurogenic actions of lithium

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Abstract

Understanding the mechanisms that regulate postnatal neurogenesis is becoming increasingly relevant since its modulation has been implicated in the pathogenesis of certain neuropsychiatric disorders. Lithium is a mood stabilizer known to increase hippocampal neurogenesis. Lithium also results in increased levels of the angiogenic factor vascular endothelial growth factor (VEGF). Since VEGF was recently shown to have neurogenic properties, we were interested to examine whether the neurogenic actions of lithium might also be accompanied by alterations in VEGF expression in the hippocampus of normal and stressed rats; the latter treatment was introduced to reproduce some of the psychopathological signs for which lithium is used therapeutically. The expression of VEGF in the hippocampus in stressed animals was lower than that in controls, but the effect of stress was significantly attenuated in animals concomitantly receiving lithium. Double staining for VEGF and specific markers for immature neurons, mature neurons and astroglia revealed that immature neurons were most sensitive to the VEGF-inhibiting effects of stress. Confirming the involvement of a known regulatory pathway in these actions of lithium, we demonstrated that lithium co-administration prevented the stress-induced upregulation of glycogen synthase kinase-3β (GSK-3β) and down-regulation of β-catenin expression; GSK-3β is a known primary lithium target and its inhibition by this mood stabilizer leads to an upregulation of β-catenin and subsequently, an increase of VEGF. Our results suggest that the neurogenic actions of lithium, and possibly its therapeutic efficacy as a mood stabilizer also, are mediated by VEGF.

Postnatal neurogenesis occurs mainly in the subventricular zone of the lateral ventricles and in the hippocampal dentate gyrus. In the hippocampal dentate gyrus, postnatal cell proliferation takes place in clusters associated with vascular niches [13]. Accordingly, a coordinated interaction between angiogenesis and neurogenesis has been proposed [8], with vascular endothelial growth factor (VEGF), a potent angiogenic factor, ranking high in the list of agents that stimulate adult neurogenesis [5]. Besides promoting cell proliferation [5], VEGF also appears to support the proneuronal differentiation of newly-born cells [11] and has been implicated in the migration of immature neuroblasts [24]. Importantly, VEGF expression has been demonstrated in astrocytes [4], neurons [7] and neuronal progenitor cells [10], all of which are endowed with VEGF receptors [10, 23].

Lithium has been one of the mainstay treatments of bipolar disease for several decades [16]. Besides modulating neuronal proliferation [3], lithium is also known to influence apoptosis (for review see [14]. Lithium's modulation of neuronal cell fate occurs through diverse downstream cascades, including inhibition of glycogen synthase kinase3β (GSK-3β) [19] an enzyme which, by inhibiting β-catenin [12] induces apoptosis and decreases neurogenesis. Recently, lithium was shown to increase myocardial levels of VEGF following an ischemic insult [6]. The increase in VEGF levels was paralleled by decreases in GSK-3β and increases in β-catenin levels. Interestingly, β-catenin itself modulates VEGF gene expression [18].

In order to better understand the neuroplastic actions of lithium, we here analyzed the influence of the mood-stabilizer on the VEGF mRNA levels and immunoreactivity in the hippocampus of rats submitted to chronic mild stress (CMS); results were compared to expression profiles in stress-free animals. We also examined whether CMS and lithium modulation of VEGF expression is confined to specific cell populations, namely in newly-acquired neurons, mature neurons and astrocytes. Finally, we examined hippocampal GSK-3β and β-catenin mRNA expression levels to confirm involvement of the canonical VEGF signalling pathway in the cellular adjustments to stress and lithium treatment.

Thirty six male Wistar rats (Charles River, Barcelona, Spain) were used in this study. Animals were housed under standard laboratory conditions (12h light cycle; 22° C, 55% humidity; food and water available ad libitum). Experiments were conducted in accordance to local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and

experimentation. All animals (4 weeks old) were divided into control (Cont) and chronic-mild stress (CMS) groups.The latter group was submitted for 14 days to a CMS protocol. The CMS protocol [21] comprised a series of different insults (confinement to a restricted space, damp bedding, water deprivation, exposure to empty bottle, tilted cage, food deprivation, exposure to restricted food and reversed light/dark cycle) that were changed daily, according to a weekly rotation plan [17]. Subgroups of Cont and CMS rats (n=9 per experimental group) were injected intraperitoneally with 2.5 mEq/Kg body weight of lithium chloride (Sigma, St. Louis, MO) (Li and CMS+Li, respectively) during the same period of time; the remaining Cont and CMS animals received saline injections. Intraperitoneal administration of lithium was preferred to chow laced with lithium to assure consistent levels of the drug. All animals were maintained on 0.9% sodium chloride in drinking water. Blood was collected from all animals at the end of the experimental procedure, > 12 h after the last exposure to stress and lithium injection; the blood sampling took place between 3 and 5 p.m. during the circadian rise in corticosterone secretion. The mean plasma levels of lithium in drug-treated animals were 0.60 (± 0.005) mmol/L, indicating therapeutic relevance.

Animals were sacrificed by rapid decapitation, the brains were carefully removed and the hemispheres separated. Right hemispheres (used for mRNA determinations) were snap-frozen in liquid nitrogen., whereas the left hemispheres (used for immunohistochemistry) were postfixed in 4% paraformaldehyde (PFA) for 48h, embedded in paraffin, cut in coronal sections of 5 μm, and mounted on non-coated glass slides.

In order to determine VEGF, GSK-3β and β-catenin hippocampal gene expression, the entire hippocampus was dissected from the brain and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA synthesis was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Semiquantitative PCR reactions were performed as previously described [22]. Briefly, each PCR cycle included the following steps: 94°C for 30 s, 57 °C for 45 s and 72 °C for 60 s. A sequential series of PCR reactions using each primer pair was initially run to determine optimal annealing temperature and cycle number to ensure amplification within the exponential phase of the amplification curve both for the gene under study and for the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT). The expression level of the reference gene (HPRT) was used

as an internal standard, to which other PCR amplification products were normalized. HPRT was chosen as a reference gene since no variation in its expression was observed between groups. The oligonucleotide primers for VEGF-A, GSK-3β, β-catenin and HPRT were synthesized using the Primer3 software on the basis of the following GenBank sequences: NM_031836 (VEGF); NM_019133 (GSK-3β); NM_053357 (β-catenin); NM_012583 (HPRT). The sequences of oligonucleotide primers were: VEGF sense, GCCCATGAAGTGGTGAAGTT; VEGF anti-sense, ACTCCAGGGCTTCATCATTG; GSK-3β sense, TTGGAAATGGGTCATTT GGT; GSK-3β anti-sense, TCACAGGATCTGCTTG; β-catenin sense, GCCAGTGGATTCCGT ACTGT; β-catenin anti-sense, GAGCTTCTCCTGATTGC; HPRT sense GCAGACTTTGCTTT CCTTGG; HPRT anti-sense TCCACTTTATGACAC. The values presented are the average of 3 PCR runs for each sample.

To assess the changes in VEGF protein, the density of cells showing VEGF immunoreactivity in the dentate gyrus was estimated in representative sections of the hippocampal formation dorsal-ventral axis. Sections were deparaffinized, immersed in TBS-T (0.1%) for 5 minutes, and microwaved while immersed in citrate buffer (0.1 M) for 15 minutes. Endogenous peroxidase activity was blocked with 3% H2O2 in TBS (10 minutes) and non-specific staining was blocked with 4% bovine serum albumin (BSA) in TBS (30 minutes). Subsequently, sections were incubated for 1.5 hours with a rabbit monoclonal anti-VEGF antibody (1:100, Lab Vision, Newmarket-Suffolk, UK). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and diaminobenzidine (DAB: 0.025% and 0.5% H2O2 in Tris-HCl 0.05M, pH 7.2). Specimens were lightly counterstained with hematoxylin. The density of VEGF positive cells in the hippocampal dentate gyrus was estimated using the StereoInvestigator software (MicroBrightField, Williston, VT) to draw the areas of interest and to count the number of positive cells within those areas; results were expressed as number of cells per area. For topographic analysis, the hippocampal dentate gyrus was divided into its main subfields: molecular layer (ML), granular cell layer (GCL), sugranular zone (SGZ) and hilus (Hi).

In view of the possibility that stress and lithium might influence only selected sub-populations of VEGF-expressing cells, the association of VEGF with newly-born neurons, mature neurons and astrocytes was assessed by double staining of VEGF with doublecortin (DCX), microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP), respectively. Using the above described protocol, sections representative of the dorsal and ventral hippocampi were

double stained for VEGF and DCX (mouse anti-DCX polyclonal antibody, 1:500, Abcam, Cambrige/UK), VEGF and MAP2 (mouse anti-MAP2 monoclonal antibody, 1:200, Sigma, St. Louis, MO) and VEGF and GFAP (mouse anti-GFAP monoclonal antibody, 1:200, LabVision, Newmarkets-Suffolk, UK). For the VEGF+DCX staining protocol, the following secondary antibodies were used: anti-rabbit Alexa Fluor-568 (VEGF) and anti-mouse Alexa Fluor-488 (DCX). In the MAP2 and GFAP double staining protocols, the following secondary antibodies were used: anti-rabbit Alexa Fluor-488 (VEGF) and anti-mouse Alexa Fluor-568 (MAP2 and GFAP). All secondary antibodies were used at a concentration of 1:200 (Molecular Probes, Eugene/OR). For each animal, 50-100 DCX, MAP2 or GFAP positive cells that double stained for VEGF were analyzed using an Olympus FV1000 confocal microscope.

The results are expressed as group means + standard error of the mean (SEM). SPSS 14.0 software (SPSS Inc., Chicago/IL) was used for statistical analysis. The homogeneity of variance was confirmed by Levene's test, prior to parametric analysis. The effects of stress and lithium alone on VEGF hippocampal levels and VEGF cell expression were assessed using independent-samples t-tests, while the overall effects were examined by two-way analysis of variance (ANOVA). Statistical significance was accepted when p was ≤ 0.05.

Body weight changes, adrenal and thymus weights were analyzed to monitor the efficacy of our stress paradigm; efficacy of the protocol was reflected in findings of significant body weight loss (18%), thymus weight loss (21%), and adrenal weight increases (20%) in the CMS-treated rats (body weight: $t = 6.2$, $p \leq 0.0005$; adrenal weight: $t = -6.5$, $p \leq 0.0005$ and thymus weight: $t = 11.6$, $p \leq 0.0005$). Lithium co-administration (CMS+Li) precluded CMS effects in adrenal ($F = 8.4$; $p \leq 0.009$) and thymus weight ($F = 5.8$; $p \leq 0.03$); however an interaction between factors was also found ($F = 186$; $p \leq 0.0005$). Lithium administration to stress-free animals led to a decrease of body weight (7%) ($t = 3.09$, $p \leq 0.002$) and an increase of adrenal weight (17%) ($t = -8.6$, $p \leq 0.0005$).

CMS decreased hippocampal VEGF mRNA ($t = 2.9$; $p \leq 0.02$) (Fig. 1A and 1B); in parallel, there was a significant reduction in the number of VEGF-immunoreactive cells in all subdivisions of the dentate gyrus of animals exposed to the CMS regimen (ML: 18%, $t = 3.2$, $p \leq 0.01$; GCL+SGZ: 34%, $t = 3.1$, $p \leq 0.012$ and Hi: 29%, $t = 4.1$, $p \leq 0.002$) (Fig. 1C and 1D). Lithium

administration to stress-free animals had no influence on either VEGF mRNA or the density of VEGF-immunoreactive cells in the hippocampus. However, co-administration of lithium and CMS (CMS+Li) prevented the stress-induce reductions in VEGF mRNA ($F = 4.3$, $p \leq 0.05$) and VEGF-immunoreactivity (ML: $F = 5.8$; $p \leq 0.03$; GCL+SGZ: $F = 4.5$, $p \leq 0.05$; Hi: $F = 4.3$, $p \leq 0.05$) levels (Fig. 1A – 1 D).

Analysis of co-localization of VEGF with DCX (Fig. 2A and 2B), MAP-2 (Fig. 2C and 2D) or GFAP (Fig. 2E and 2F) confirmed that CMS led to a decrease in the number of cells producing VEGF in all three cell phenotypes (DCX: $t = 3.9$, $p \leq 0.0005$; MAP2: $t = 3.5$, $p \leq 0.003$ and GFAP: $t = 7.6$, $p \leq 0.02$), without any effect being observed when lithium was administered to stress-free animals (Table 1). Interestingly, CMS treatment led to more pronounced effects on VEGF-immunoreactivity in immature neurons (DCX) as compared to mature neurons (MAP2) and astrocytes (GFAP) ($t= 4.3$, $p \leq 0.0005$) (Table 1). Two-way ANOVA showed that administration of lithium concurrently with stress (CMS+Li) precluded the deleterious effects of CMS on VEGF producing cell numbers (DCX: $F = 21.8$, $p \leq 0.0005$; MAP2: $F = 12.0$, $p \leq 0.002$ and GFAP: $F = 10.0$, $p \leq 0.03$); moreover, a significant interaction between stress and lithium was found ($p \leq 0.003$) (Table 1).

GSK-3β and β-catenin gene expression were significantly altered by CMS but not by lithium per se (GSK-3β: $t = -5.7$, $p \leq 0.0005$; β-catenin: $t = 9.6$, $p \leq 0.0005$) (Fig. 3A to 3D). Two-way ANOVA revealed that lithium co-administration to animals undergoing the CMS procedure (CMS+Li) results in an abolition of stress-induced increases in GSK-3β levels ($F = 11.6$, $p \leq 0.003$), while also attenuating the effects of stress on β-catenin expression ($F = 32.8$, $p \leq 0.0005$). In addition, there was an interaction between stress exposure and lithium administration for both enzymes ($p \leq 0.0005$) (Fig. 3A and 3D).

There is a growing interest in the factors and mechanisms that regulate neurogenesis in the hippocampus since the demonstrations that the therapeutic effects of antidepressants of every class are accompanied by increases in the rate of neuronal birth [9, 16]. Lithium, a classical mood stabilizer has also been shown to stimulate the proliferation of hippocampal neuronal progenitor cells [2]. One of the factors that has recently attracted attention in this respect is the angiogenic factor, VEGF. The ability of this factor to stimulate cytogenesis in the vascular-stem

cell niche interface has been clearly demonstrated [13]. Interestingly, lithium was found to stimulate VEGF expression in the ischemic myocardium [6].

Chronic stress is a potent inhibitor of hippocampal neurogenesis and plasticity [3, 15]; at the same time, chronic stress can trigger mood disorders [1]. In this study, we show that chronic mild stress causes significant decreases in the steady-state levels of VEGF mRNA and immunoreactivity in the hippocampus. Our results are in accord with those of another recent study in which a different stress paradigm was used [4]. An important finding in the present report is that administration of lithium during exposure to chronic stress abolishes the deleterious effects of stress on hippocampal VEGF mRNA and immunoreactivity. This result therefore shows that, besides antidepressants [20], mood stabilizing compounds such as lithium in stressful contexts can also stimulate VEGF production. It is important to note that the stress- and lithium-induced changes in VEGF expression in this study also occur in the dentate gyrus, the neurogenic zone of the hippocampus. Further, by using double immunocytochemical labeling, we show that VEGF expression in neuroblasts (DCX-positive), mature neurons (MAP2-positive) and astrocytes (GFAP-positive) is subjected to regulation by stress and lithium.

Here it was found that stress induced an increase of GSK-3β mRNA, a kinase which lies immediately downstream from VEGF, and that lithium co-administration precluded this action. In turn, these treatments respectively decreased and increased levels of the mRNA encoding β-catenin, a signaling protein with known proliferative actions. These findings suggest that the anti-neurogenic effects of stress are mediated through VEGF's canonical signaling pathway, and that the ability of lithium to prevent the deleterious effects of stress occurs at a level proximal to VEGF production. On the other hand, since lithium did not produce any changes in hippocampal VEGF production in stress-free animals, it seems likely that lithium can only work in specific cellular milieus, e.g. those prevailing during psychopathological states. However, it should be remembered that lithium was shown to increase neurogenesis in rodents [2], thus supporting the view that lithium therapeutic actions occur through several molecular pathways. The present experiments therefore provide several new ideas to be tested with respect to understanding the cellular mechanisms through which lithium exerts its mood stabilizing effects.

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Tables

Table 1. Percentage of immature neurons (DCX), mature neurons (NeuN) and astrocytes (GFAP), double-labeled with VEGF in the hippocampal dentate gyrus.

	Cont	CMS	CMS+Li	Li
DCX+VEGF	20 (1.8)	2 (1.4)*	20 (2.6)**	21 (1.9)
MAP2+VEGF	33 (2.8)	21 (1.3)*	36 (1.3)**	32 (1.9)
GFAP+VEGF	64 (1.6)	54 (3.9)*	67 (1.5)**	58 (1.8)

Groups: control (Cont), chronic mild stress (CMS), chronic mild stress lithium (CMS+Li) and lithium (Li) Values are expressed as means (+ SEM).* significant to Cont group; ** significant to CMS group.

Figures

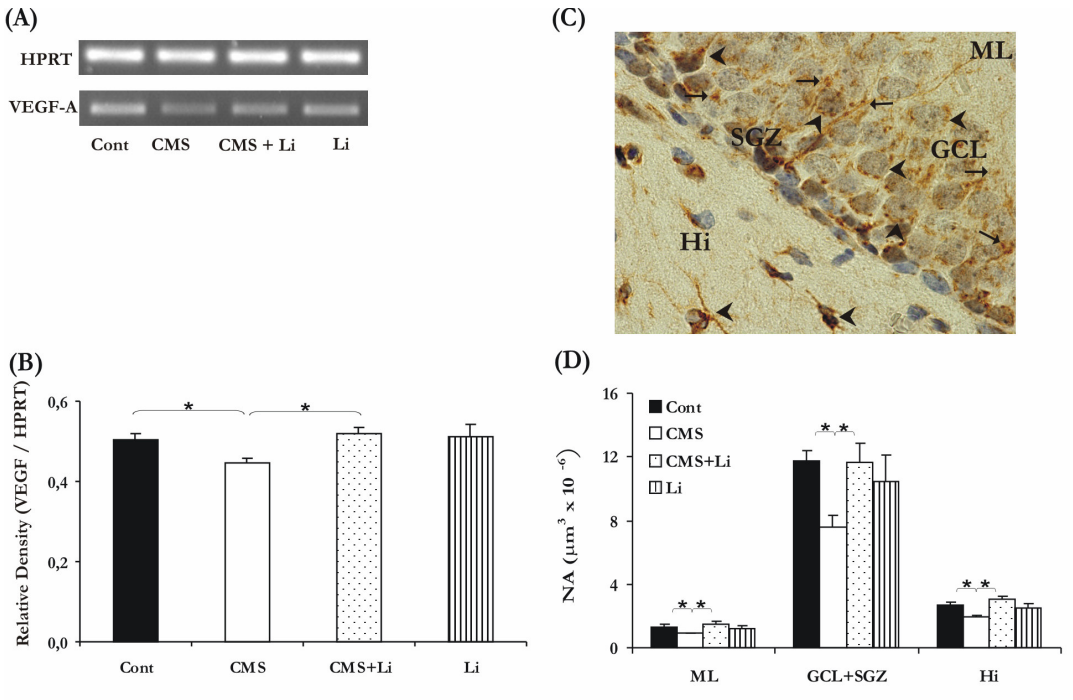

Fig. 1. Photomicrograph illustrating an example of VEGF mRNA analysis using semi-quantitative PCR (A). Graphic representation of VEGF gene expression in control rats (Cont) and rats subjected to chronic mild stress (CMS), chronic mild stress + lithium or lithium (Li). (B). CMS significantly downregulated VEGF mRNA levels ($p \leq 0.02$) (A and B), an effect prevented by the concomitant administration of lithium (CMS+Li) ($p \leq 0.05$) (A and B). Administration of lithium alone (under stress-free conditions) did not result in changes in VEGF mRNA levels (A and B). Photomicrograph illustrating VEGF immunoreactivity in the hippocampal dentate gyrus areas (molecular layer (ML), granule cell layer (GCL), subgranular zone (SGZ) and hilus (Hi). Cells were considered positive to VEGF immunostaining (arrowheads) when the fraction of the stained area was ≥ 1.0 x 10-4. Arrows indicate non counted staining; scale bar: 100µm (C). Graphic representation of the number of VEGF positive cells in the main subdivisions of the hippocampal dentate gyrus (molecular layer (ML), granule cell layer (GCL), subgranular zone (SGZ) and hilus (Hi)) (D). Lithium abolished the ability of CMS to decrease the density of VEGF positive cells in all subdivisions of the dentate gyrus ($p \leq 0.02$) ($p \leq 0.05$); lithium administration to stress-free animals did not significantly alter the density of VEGF expressing cells (C-D).

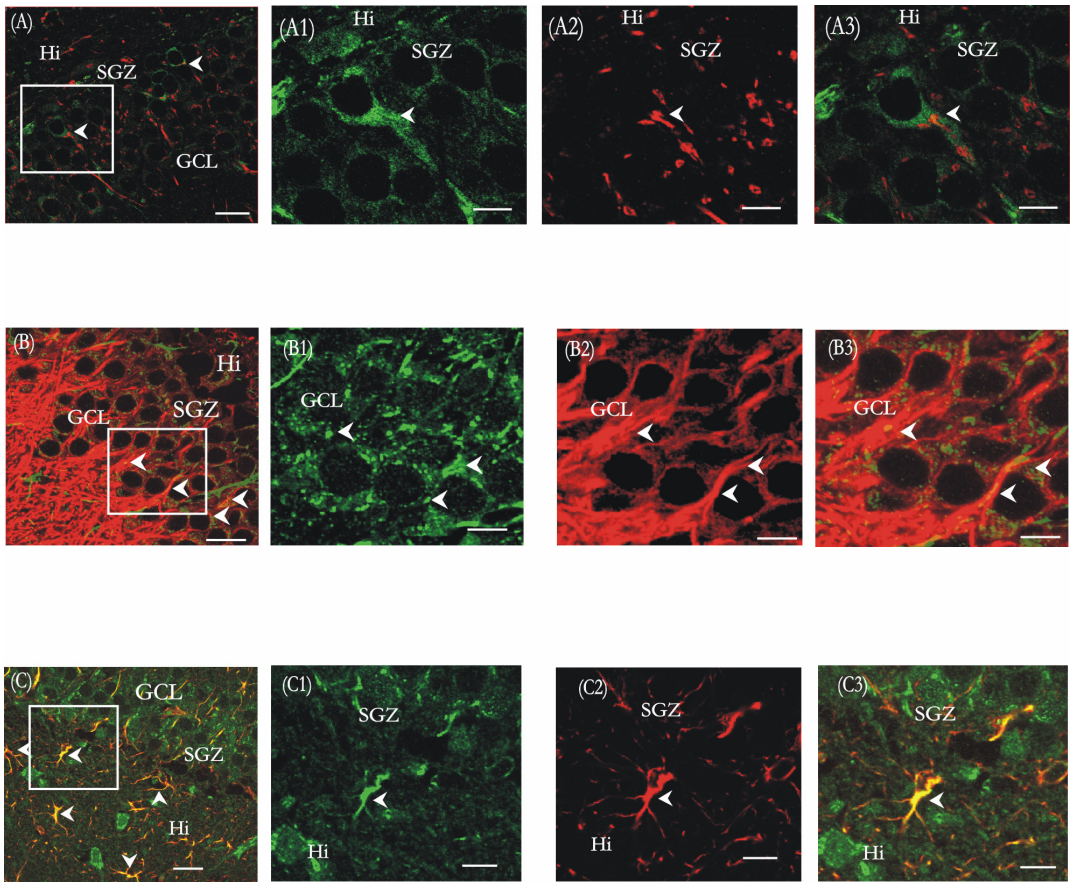

Fig.2. Confocal photomicrographs illustrating co-localization (arrowheads) of DCX (green) and VEGF (red) (A) (A1 green channel, A2 red channel; A3 merged); MAP2 (red) and VGF (green) (B) (B1 green channel, B2 red channel; B3 merged); and GFAP (red) and VEGF (green) (C) (C1 green channel, C2 red channel; C3 merged) in the hippocampal dentate gyrus areas (granule cell layer (GCL), subgranular zone (SGZ) and hilus (Hi)). Scale bar: 50μm (A, B and C) and 5μm (magnifications).

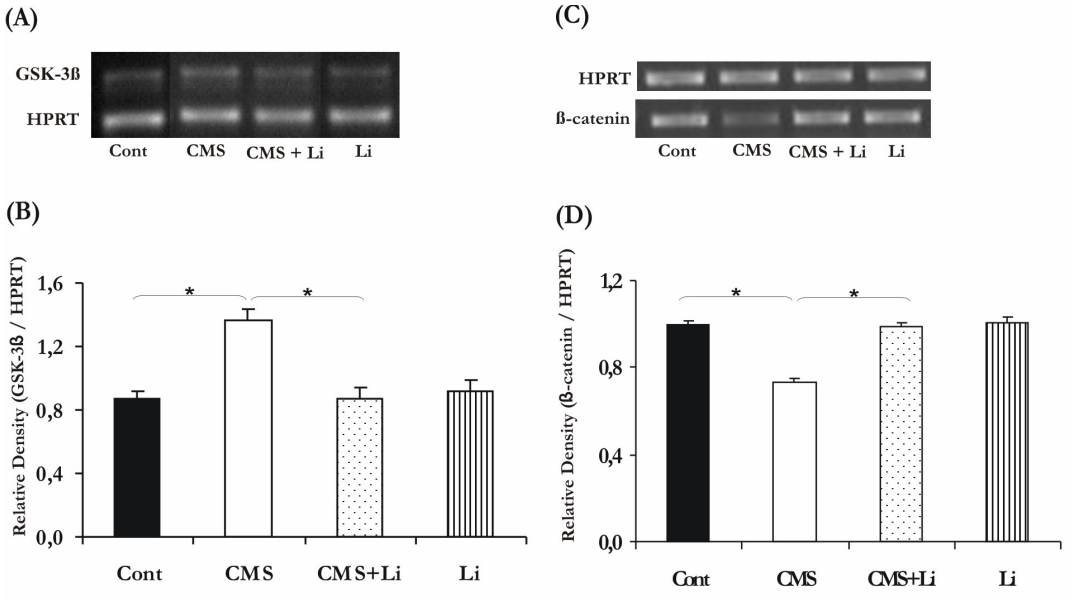

Fig.3. Photomicrograph illustrating an example of a GSK-3β mRNA analysis by semi-quantitative PCR (A). Comparisons of GSK-3β gene expression between control (Cont), chronic mild stress (CMS), chronic mild stress + lithium (CMS+Li) and lithium (Li) groups, revealed significantly increased levels of GSK-3β in the CMS but not in the CMS+Li group ($p \leq 0.003$) (B). Photomicrograph illustrating an example of a result from β-catenin mRNA analysis (C). Graphic representation of β-catenin gene expression (D). Data indicated a decrease of β-catenin mRNA levels with CMS ($p \leq 0.0005$) (C and D) that was blocked when lithium was given during the period of stress (CMS+Li) ($p \leq 0.0005$) (C and D); Lithium administration to stress-free animals did not alter GSK-3β or β-catenin mRNA levels (A to D).

Chapter 3

Results and Discussion

3. Results and Discussion

Adult neurogenesis has generated great interest as a possible therapeutic target for some brain pathologies. Despite numerous studies, its function and regulatory pathways are still not totally clear, which limits a possible therapeutic use. In the present thesis we studied the influence of stress and corticosteroid imbalances in the rate of adult neurogenesis, as well as the pathways through which these regulatory actions might operate. Since it is believed that the maintenance of the normal brain structure and function results from balanced cell turnover, the densities of hippocampal neurogenesis *versus* cell death, by apoptosis, were herein analysed.

Rates of hippocampal cell birth and death were first studied in basal conditions; the observation of gradients in the topographic distribution of neurogenesis and apoptosis in the hippocampal dentate gyrus is presented and discussed in chapter 2.1. In order to assess the effects of modulatory mechanisms in hippocampal cell turnover, neurogenesis and apoptosis were scrutinized in basal and after neuronal insults. Stress and stress hormones, which are known to decrease proliferation and increase apoptosis (Gould et al., 1991a; Gould et al., 1991b), are thought to play a role in depression (Gibbons et al., 1962; Sonino et al., 2001; Gillespie et al., 2005). Thus, the balance between proliferation and apoptosis and the topographic distribution of both events were analyzed after exposure to a chronic mild stress, an animal model of depression (chapter 2.2). Moreover, the discrimination of the role of each corticosteroid receptors (MR and GR), was assessed after administration of corticosterone, dexamethasone or after prolonged exposure to adrenalectomy (chapter 2.3). The impact of stress and stress hormones on the expression of VEGF, a growth factor involved in neurogenesis and angiogenesis, was also assessed (Chapter 2.4). Finally, the ability of lithium (a mood stabilizer with antidepressant propoerties) to prevent the stress-induced effects on depressive behaviour, neuronal cell fate, and in the associated molecular pathways was also analysed (chapters 2.2, 2.3 and 2.4).

3.1 Gradients of neurogenesis and apoptosis

Hippocampal cell birth and death do not occur in a homogeneous manner, but rather display topographic gradients (Chapter 2.1). Although certain gradients of neurogenesis and apoptosis overlap (in both events, ventral > dorsal), the majority are distinct. As expected, we observed that the higher density of neurogenesis occurs in the SGZ; within the SGZ, the intermediate

subdivision of the infrapyramidal of dentate gyrus displayed the highest index of cell proliferation. In contrast, the higher density of apoptosis occurs in the extreme subdivision of the suprapyramidal blade of the SGZ. Importantly, apoptosis indexes were higher in the left hippocampal dentate gyrus (Chapter 2.1).

Telencephalic lateralization is thought to reflect the developmental specialization of functions within hemispheres (Toga and Thompson 2003). It is believed that, in most humans, the left hemisphere is responsible for language processing, while the right is more specialized in spatial and emotional functions (Rosen, 1996). These hemispheric differences are mainly functional but also reflect structural specifications, that start to develop early in neurodevelopment (Molfese et al., 1975; Giedd, et al., 1996; Utsunomiya et al., 1999; Vertstynen et al., 2001; Sowell et al., 2002), but are still operating in the adult brain (Diamond et al., 1982; Papanicolaou et al., 2002; Maguire et al., 2000; Pedraza et al., 2004; Maguire et al., 2006). In normal adult subjects hemispheric differences are observed in some brain structures, as peri-sylvian cortices (Toga and Thompson, 2003), amygdala (Pedraza et al., 2004) and hippocampal formation (Diamond et al., 1982; Pedraza et al., 2004).

Considering that these asymmetries are also a result of changes in total volumes and neuronal numbers, all the events related with cell birth and/or death are significant for the creation of asymmetric cortical areas (Rosen, 1996). The left *vs* right hippocampal formation discrepancies in the apoptosis rate herein observed becomes very interesting, as it may contribute to explain structural and functional differences in the adult hippocampal formation. The function of the hippocampal formation seems to differ from the left to right hemisphere: while the right hippocampal formation is largely associated with nonverbal tasks (Papanicolaou et al., 2002), as spatial navigation (Maguire et al., 2000; 2006), left hippocampal formation is more related with verbal function (Papanicolaou et al., 2002). Taking into account that the main projections from the hippocampal formation to the PFC are mainly ipsilateral (Ferino et al. 1987; Jay et al., 1989; Jay et al., 1991), it is interesting to note that the left prefrontal cortex (PFC) is also associated with semantic conditioning (Fletcher et al., 1997). Importantly, it was observed that the left PFC, which is implicated in the regulation of the HPA axis (Maclullich et al., 2006), is more sensitive to glucocorticoid actions than the right PFC (Cerqueira et al., 2005).This increase susceptibility together with the lower volume of the left hippocampal formation (Pedraza et al., 2004),

strengthens the relevance of our observation of a higher apoptotic index in the left hippocampal dentate gyrus in basal and pathological (including corticosteroids imbalances) conditions. Hippocampal asymmetries assume a great importance, as psychiatric disorders associated with an impairment of HPA axis function, as depression, have a higher incidence in subjects with smaller hippocampal volumes (Mervaala et al., 2000), but also because the intensity of depressive symptoms are related with the volume of left hippocampus (Bremner et al., 2000).

Despite these differences in left vs right hippocampal formations, some authors propose a "unitary processing framework" for the hippocampal formation (Hoz et al., 2003). This hypothesis sustains that the hippocampal intrinsic circuit is similar along dorsal-ventral axis and, thus, the behavioural differences that arise from hippocampal lesions are largely due to effects in memory processes, as consolidation of learning rate (Hoz et al., 2003). However, functional differences of distinct areas of the hippocampal formation were described, with ventral and dorsal hippocampi having different roles, namely in memory and in environmental information integration: dorsal (septal) hippocampus is involved in spatial learning, while ventral (temporal) contributes more significantly to nonspatial tasks of learning (Moser and Moser, 1998). In accordance with the functional differences between dorsal and ventral hippocampal formation we here observed the occurrence of neurogenesis and apoptosis' gradients throughout the hipocampal longitudinal axis, with higher densities of cell birth and death in ventral hippocampal dentate gyrus. A higher cell turnover may reflect increased neuroplasticity associated with learning and memory and the capacity to process new information (Chambers et al., 2004). Therefore, one might speculate that the increased rates of neurogenesis and apoptosis observed in the ventral hippocampal dentate gyrus may be related with a higher renewal of nonspatial information, whereas the lower cell turnover of dorsal hippocampal dentate gyrus may reflect the more static nature of spatial information.

The hippocampal dentate gyrus is normally assumed as uniform in terms of structure, organization and function (Amaral et al., 1978; Frotscher et al., 1992; Tamamaki and Nojyo, 1993; Frotscher et al., 1994). However, this homogeneity may not be so generalized as i) the axon plexus is greater in the suprapyramidal blade (Nitsch and Leranth, 1993); ii) there are higher numbers of "basket" cells and GABAergic neurons associated with the suprapyramidal blade (Ribak and Peterson, 1991); iii) the suprapyramidal blade is more sensitive to

adrenalectomy (Sloviter; 1989) and alcohol (Cadete-Leite et al., 1997); iv) the infrapyramidal blade is more vulnerable to hypoxia (Hara et al., 1990) and aging (Simonyi et al., 2000); and v) the stimulation of the infrapyramidal blade seems to induce a higher response of CA3 pyramidal cells than that resulting from suprapyramidal blade stimulation (Scharfman et al., 2002). Therefore, the differences in topographic distribution of neurogenesis and apoptosis between the supra and infrapyramidal blade herein reported are likely to contribute to the asymmetries in the structure and functionality of this two blades of the granule cell layer. The increased neuronal death observed after adrenalectomy and alcohol in the suprayramidal blade (Sloviter; 1989; Cadete-Leite et al., 1997), may be related to the higher basal susceptibility of this blade to apoptosis (Chapter 2.1). On the other hand, the higher rate of neurogenesis observed in the infrapyramidal blade (Chapter 2.1) possibly reflects the higher activation of this blade as a result to its higher efficacy after stimulation (Scharfman et al., 2002); importantly, it is also plausible to admit that such difference might promote a better integration (including acquisition and consolidation) of new information (Chambers et al., 2004).

These first results (Chapter 2.1) showed that studying only neurogenesis or apoptosis may lead to misinterpretations, since one event might be compensated by the other. This emphasizes the relevance of assessing the final cell balance when analysing cell fate in hippocampal formation. Moreover, the finding of specific gradients of neurogenesis and apoptosis indicates that the dentate gyrus should not be viewed as a uniform structure and that different gradients of neurogenesis and neuronal apoptosis might explain different vulnerability of the blades of the dentate gyrus to specific insults.

3.2 Stress and corticosteroid milieu imbalances modulate hippocampal cell turnover

The modelling of human depression using animal models is extremely difficult. Many different models have been proposed but, unfortunately, none of the existent models is able to mimic all the components of the disorder; to this fact, it is not strange the subjective nature of many of the symptoms. Therefore, this handicap must be considered whenever using animal models of depression. One of the models that presents greater validity is the exposure to stressors (Berton and Nestler, 2006). However, also in this model the debate of the meaning of stress-depressive

effects occurs, because it is based on the exposure to stressful environments of animals that do not have a higher susceptibility to the disorder (Berton and Nestler, 2006). The model herein used (chronic mild stress, CMS) has the advantage of using natural stressors and being prolonged in time (Willner et al., 1987); this protocol triggers several physiological consequences that are also present in depressive patients, including anhedonia (lack of pleasure) and learned helplessness (Nestler et al., 2002). The assessment of depressive-like behaviour induced by CMS relies on two measurements to evaluate learned helplessness (a depression-like behaviour characteristic) in the forced-swimming test (FST): latency to immobility and immobility time. This test is one of the most used and its main advantages rely in the link between cognitive function (learning) and "neurovegetative changes", which provides an integrated perspective of depressive symptoms (Nestler et al., 2002). Despite the clear benefits, of both the model and endpoints herein used, one must considered some potential pitfalls (Nestler et al., 2002): i) it is still a matter of debate if learned helplessness is a better model of post-traumatic stress than of depression; ii) protocols that affect mobility or stimulate the animals may lead to false conclusions in the FST; iii) single administrations of antidepressants decrease FST immobility time, even tough their clinical effects require weeks of treatment. In summary, there is no perfect animal model of depression and thus one must choose the best amongst the available; obviously, care must be taken in order to guarantee the validity of the results.

Considering that depression seems to be a neuroplastic rather than a neurodegenerative disorder (Czéh and Lucassen, 2007) the changes induced by CMS in hippocampal dentate gyrus final cell balance might be view as relevant to CMS-induced depressive-like behaviour (Chapter 2.2). Although neither neurogenesis nor apoptosis changes *per se* can explain hippocampal atrophy in depression (Czéh and Lucassen, 2007), they may contribute to alterations in the hippocampal connectivity and, consequently, to modifications in its functions (Joëls et al., 2004). Impairments in hippocampal function are known to be associated with memory and cognition deficits (Sousa and Almeida, 2000; Belanoff et al., 2001), which are symptoms presented not only by subjects suffering from Cushing's disease or exposure to corticotherapy, but also by most depressive (Brown et al., 1999; Austin et al., 2001; Marvel et al., 2004) and bipolar disorder patients (Quarishi and Frangou, 2002). Interestingly, cognitive impairments worsen with the severity of these disorders (Sax et al., 1995). In further support of a link between depression and cognitive impairments, structural and functional abnormalities of other brain regions, besides the

hippocampal formation, namely the PFC (Drevets et al., 1997; Mayberg et al., 1997; Hirayasu et al., 1999; Rogers et al., 2004) and amygdala (Drevets et al., 2003) were reported in depressive patients presenting cognitive deficits (Savitz et al., 2005). These studies point to a depression-associated impairment of the limbic system (Austin et al., 2001). Considering that i) corticosteroids are potent modulators of the central nervous system, namely the limbic system (Sousa and Almeida, 2002; Sousa et al., 2007), ii) hypercortisolemia is present some depressive patients (Gibbons et al., 2001), and iii) patients with Cushing's syndrome also display depressive symptoms (Sonino et al., 2001), high levels of corticosteroids are certainly a major candidate to induce these structural, functional and cognitive impairments associated with mood disorders (Brown et al., 1999; Sapolsky et al., 2000a; Sousa et al., 2007).

To ascertain if the effects of stress are due to increased corticosteroids we administrated exogenous corticosterone to another set of animals. The exogenous administration of high levels of Cort is a common model used to study effects of stress in the central nervous system. However, it does not truly mimics the entire set of changes involved in the stress response, namely the stress activation of the HPA axis (e.g. increased secretion levels of CRF and ACTH in the hypothalamus and pituitary, respectively). However, either as part of the stress response or after exogenous administration, corticosteroids induce their effects through activation of MR and GR, which have distinct cellular effects (for review see Sousa et al., 2007). The next step was to discriminate if the effects of corticosteroids were acting through GR receptors; for this proposed, we used exogenous administration of specific agonists of GR, such as dexamethasone (Dex).

At low concentrations Dex has a small penetration in the brain due to the action of the mrd1a-encoded P-glycoproteins (de Kloet et al., 1997; Meijer et al., 1998). However, this barrier is not able to completely prevent penetration of higher doses of Dex, and ultimately the hormone gets into the brain. Because GR activation induces a negative-feedback in the HPA axis activity, this will abolishes the endogenous production of corticosteroids, leading to a non-occupancy of MR (Reul et al., 1987; Spencer et al., 1990; Karssen et al., 2005).

Although Dex influenced both parameters of the FST, Cort only affected immobility time (Chapter 2.3). These results support the view that corticosteroids play a role in depression (Gillespie and Nemeroff, 2005). However, the detailed analysis of animals treated with Cort also reveals that

high levels of corticosterone *per se* are not sufficient to induce the complete stress-induced depressive-like phenotype. Therefore, other concomitant upstream events (such as the increase of vasopressin and CRF levels) must be involved in stress-induced depression. In fact, besides its role as a trigger of ACTH production in the pituitary, CRF also acts as a neurotransmitter in the CNS (Carrasco and Van der Kar 2003). The role of CRF in depression is favoured by reports of increased CRF brain levels in a significant number of depressive patients (Gillespie and Nemeroff, 2005) and by the antidepressant actions of CRF-1 antagonists (Louis et al., 2006). In addition, vasopressin is a neurotransmitter also known to act in limbic structures (Debiec, 2005; Carrasco and Van der Kar 2003). Studies revealed that antidepressants stabilized the levels of vasopressin (Keck et al., 2003; Scott and Dinan, 2002) and that antagonists of vasopressin receptor 1b have antidepressant effects (Salome et al., 2006; Louis 2006).

In order to assess the mechanism through which the modulatory actions of stress in neurogenesis and apoptosis occurred, we studied the impact of exogenous corticosteroids in the final hippocampal cell balance. Interestingly, the topographic gradients of neurogenesis and apoptosis seen in basal conditions were maintained after modifications of corticosteroid milieu. Corticosteroids are known to decrease neurogenesis (Gould et al., 1991a) and increase apoptosis Gould et al., 1991b); the present data corroborated these observations (Chapter 2.3). Concomitantly, we also observed that Dex induced higher apoptotic indexes than Cort (Chapter 2.3), which further supports the view that MR activation is neuroprotective (Almeida et al., 2000). Considering that the hippocampus is involved in the negative-feedback control of the HPA axis (Jacobson and Sapolsky, 1991), this impaired cell balance, besides inducing memory and cognitive deficits (Sousa and Almeida, 2002), may contribute to a deregulation of the HPA axis, which leads to its hyper-function and, consequently, to a higher damage of the hippocampal formation. Once more, the glucocorticoid cascade hypothesis is "revisited". However, the sequence of these events is again not clear. Is it HPA axis deregulation that induces a reduction of hippocampal final cell balance, or do hippocampal structural and functional impairments lead to a hyper-activity of the HPA axis? Most probably different contexts might lead to distinct trigger events but, ultimately, they might converge in this cascade!

Besides receptor activation, also its unoccupancy is used to study corticosteroid receptors actions. The removal of the adrenal glands (adrenalectomy (ADX)), with the consequent

unoccupancy of both MR and GR, is generally used to study the effects of corticosteroids (in particular MR, due to the affinity properties of the receptors). Although the absence of corticosteroids was predict to be neuronal protective (Landfield et al., 1981) it was rapidly discovered that ADX induces massive neuronal death, namely in the dentate gyrus (Sloviter et al., 1989). Subsequently, it was observed that besides inducing apoptosis, ADX also increases hippocampal neurogenesis (Cameron and Gould, 1994). We observed that ADX induced a depressive-like behaviour and decreased hippocampal final cell balance. However, contrary to Cort and Dex protocols, this reduction was a consequence of the marked increase of apoptosis, that clearly outweighed the one of neurogenesis (Chapter 2.3). These results are in accordance with the previously described ADX effects in hippocampal cell proliferation (Cameron and Gould 1996) and death (Sloviter et al., 1989; Sapolsky et al., 1991), and showed that a depressive-like behaviour can occur despite increased hippocampal neurogenesis. In other words, these data also shows that impaired neurogenesis *per se* is not an essential factor to the induction of a depressive-like behaviour. In summary, our results indicate that the final cell balance is a better indicator of alterations in hippocampal plasticity associated with depressive-like behaviour, than neurogenesis alone.

The activation of MR was confirmed to be of great importance for hippocampal structure and function. Indeed, the link between hippocampal MR and depressive behaviour was based on several observations: i) MR are mainly found in the hippocampus (Joels and de Kloet, 1994), ii) increases in MR binding are amongst the first alterations observed after one week of antidepressant treatment (Reul et al., 1993, 1994), iii) co-administration of a MR antagonist with antidepressant blocks treatment response (Holsboer, 2000) and iv) MR, but not GR, gene disruption induces hippocampal granule cell degeneration (Gass et al., 2000). However, differential activation of MR (Ficher et al., 2002; Montarton et al., 2003) and GR (Montarton et al., 2003) in an ADX context showed that both receptors are able to influence ADX-induced increase in apoptosis and proliferation, indicating that corticosteroids actions in cell birth and death occur through both receptors. This was confirmed by Crochemore and colleagues (2005) observations, which showed that GR can directly induce hippocampal apoptosis, an event that is modulated by MR. The relevance of MR and GR in proliferation and apoptosis supports the proposal that deregulation of MR/GR balance has a crucial role in the pathogenesis of depression (Holsboer, 2000). As a note, it is relevant to mention that the role of MR/GR in depression led to

the use of anti-corticosteroids (steroid inhibitors and GR antagonist – mifepristone) to treat depressive symptoms; unfortunately, however, these treatments proved to be either inconclusive or to induced severe side effects (Young, 2006).

3.3 The preventive influences of lithium

Lithium has been used in clinical practice as a mood stabilizer, for the treatment of bipolar disorder for several decades. This drug is known to prevent pathological mood alternation between mania/hypomania and depression. However, lithium has also antidepressant capacities and, in particular cases, is used as an adjuvant to classic antidepressants. Lithium, as well as classical antidepressants, regulates GR mRNA (Semba et al., 2000), and increases both neurogenesis (Chen et al. 2000) and the expression of anti-apoptotic proteins (Manji et al., 2000; Zhou et al., 2005).

An important characteristic of antidepressants is their inability to improve mood in healthy subjects (Berton and Nestler 2006). Interestingly, this was confirmed in the present studies; lithium administration *per se* not only did not improve the learned helpleness behaviour, as it increased FST immobility time, as previously described (Tomasiewicz et al., 2006) (Chapters 2.2 and 2.3). However, in parallel, lithium administration to stress-free rats increased both proliferation and apoptosis (Chapter 2.2). The final cell gain was higher than in controls in all hippocampal dentate gyrus areas, except in the GCL, where mature granule cells reside. This data supports the view that lithium displays neuroplastic (Chen et al. 2000, Manji et al. 2000, Hall et al., 2000) and protective roles (Wada et al., 2005) in proliferating cells, but does not induce its migration and thus, it does not positively influence the function of hippocampal formation, as seen in spatial memory tasks (Bessa et al., unpublished data) or in depressive-like behaviors (Chapters 2.2 and 2.3).

A completely different picture emerges from the administration of lithium to stressed animals. Indeed, in the present work we demonstrate that lithium co-administration prevented the induction of depressive-like behavior and hippocampal cell balance effects triggered by the different neuronal insults (CMS, Cort, Dex and ADX) (Chapters 2.2 and 2.3). These data supports the existence of an association between stress and depressive-like behaviour through

hippocampal impairments. In accordance, previous studies have reported a link between stress, hippocampal structure and hippocampal-dependent learning and memory impairments (Sousa et al., 2000). In addition, it was shown that lithium treatment prevents stress-induced decrease of dendritic length (Wood et al., 2004) and spatial memory performance (Vasconcellos et al., 2003). From these findings, a new question emerged: are the precluding behavioural and cell fate effects of lithium a result of a normalization of corticosteroid levels (as confirmed by corticosterone plasmatic levels), or is this hormonal normalization a consequence of the regulation of the final cell balance and parallel increase of GR mRNA (Semba et al., 2000)? Our results in animals receiving Cort, Dex, or even after ADX, showed that lithium prevented the appearance of behaviour impairments and disruption of hippocampal final cell balance, even in the absence of a normal corticosteroid level (Chapter 2.3). These seem to point to the conclusion that lithium behavioural and plastic actions are independent of the restoration of corticosteroid milieu; however, its preventive effects in the hippocampal final cell balance may, subsequently, contribute to the normalization of corticosteroid levels.

At this point one might think that the link between adult hippocampal neurogenesis and depressive-like behavior could be the key for the appearance/maintenance, and even the resolution, of mood disorders (Santarelli et al., 2003). However, other results of our group (Bessa et al. 2007), showed that decreased neurogenesis *per se* is not sufficient to induce the alterations in hippocampal activity that lead to a depressive-like behaviour. Moreover, that study also shows that an increased neurogenesis is not required for antidepressant actions. This information redirects the field to alternative mechanisms. In this line, it is crucial to remember that antidepressants act mainly by regulating monoaminergic pathways, but recently, serotonin and antidepressants were also shown to inhibit GSK-3β (Li et al., 2004; Roh et al., 2005), indicating new targets to these drugs. Additionally, lithium, a GSK-3β inhibitor, was reported to increase serotonin synthesis (Price et al., 1990), release (Treiser et al., 1981) and neurotransmission (Bauer et al., 2003), emphasizing the link between all these factors. It seems, therefore, essential to assess some of the molecular pathways through which drugs with antidepressant effects may operate.

3.4 Molecular pathways involved in lithium prevention of stress/corticosteroids-induced effects

Even though being clinically used for such a long time, the molecular mechanisms through which lithium acts are not totally understood. Several cellular effects of lithium have already been unveiled, including modulation of the inositol signalling pathway (Berridge et al., 1989; York et al., 1995) and inhibition of the enzyme GSK-3 (Stambolic et al., 1996). Although differences of GSK-3 levels between frontal and occipital cortex form bipolar patients and controls have not been detected (Lesort et al., 1999; Agam et al., 2003), GSK-3β is a prime candidate for this study as: i) it has been associated with corticosteroids actions (Sotiropoulos et al., 2006), ii) it was related with reduction of neurogenesis, through inhibition of β-catenin (Yost et al., 1996; Orford et al., 1997), iii) its specific inhibitors have antidepressant actions (Gould et al., 2004b; Kaidanovich-Beilin et al., 2004 and present study), and iv) Akt, one of GSK-3β inhibitors, was reported to be actively decreased in the occipital cortex of depressed suicide patients (Hsiung et al., 2003). However, we observed that GSK-3β levels were increased by CMS and Dex administration and that lithium administration concomitant to CMS or Dex, prevented this effect (Chapters 2.2 and 2.3). Considering also that, as formerly discussed, lithium co-administration prevented CMS- and Dex-induced depressive-like behaviour, these results are in accordance with a previous study, which showed that GSK-3β +/- transgenic mice have a reduced FST immobility time (O'Brien et al 2004), and support the view that GSK-3β might be altered in mood disorders, including depression.

As a result of GSK-3β increase, β-catenin was decreased by CMS (Chapter 2.4). These observations are in agreement with previous reports showing that stress and glucocorticoids induce an increase of GSK-3β (Lin et al., 2006; Sotiropoulos et al., 2006) and a decrease of β-catenin (Yost et al., 1996; Lie et al., 2005; Lin et al., 2006). Taking into account that CMS Cort and Dex also decrease final cell balance, this data supports an essential role for this Wnt pathway protein in the regulation of cell proliferation, differentiation (Lie et al., 2005; Hirsch et al., 2006) and death (Li et al., 2002). Therefore, it is possible to infer that CMS effects in cell fate and behaviour may occur also through modulation of GSK-3β levels, which, among other known actions, induces a decrease of β-catenin.

As previously referred, apoptosis seems to have an important role in hippocampal plasticity. Both lithium and corticosteroids are known to influence the expression of pro- and anti-apoptotic proteins, namely from the Bcl_2 family (Stambolic et al. 1996; Manji et al., 2000, Almeida et al., 2000). BAG-1 expression was previously reported to be increased by lithium (Zhou et al., 2005) and to modulate GR function (Schneikert et al., 1999). We observed that BAG-1 levels were decreased in CMS and Dex-treated animals (Chapters 2.2 and 2.3), which is in good correlation with the increase density of hippocampal dentate gyrus apoptosis in these experimental conditions (Chapter 2.3). Interestingly, BAG-1 levels were preserved in Cort-treated rats. These results are in accordance with the described pro-apoptotic actions of corticosteroids (Sousa at al., 1999; Almeida et al., 2000; Crochemore et al., 2005) and with the protective role of MR when compared to GR activation, which increases Bax/Bcl-2 ratio (Almeida et al., 2000). Again, our studies reveal that lithium co-administration (CMS+Li, Cort+Li and Dex+Li) precluded the stress and Dex effects in BAG-1 expression, supporting a protecting role of lithium in cell fate events (Chapters 2.2 and 2.3). These results open the possibility that the depressive-effects of stress and stress hormones may occur not only through a deregulation of monaminergic pathways (for review see van Praag 2004) but also through anti-proliferation and pro-apoptotic mechanisms. They also indicate that the therapeutic actions of lithium, and other drugs with mood-stabilizing effect properties, operate also through mechanisms promoting of cell survival.

As expected from the variation of hippocampal GSK-3β expression, the levels of synapsin-I, a pre-synaptic protein that is a down-stream target of GSK-3β (Hall et al. 2000), were decreased in hippocampus of CMS and Dex treated rats (Chapter 2.2 and 2.3). This reduction in synapsin-I levels by stress and corticosteroids supports previous studies that showed a decrease of another pre-synaptic protein (synaptophysin) by stress (Thome et al., 2001; Xu et al., 2004). It also indicates a possible reduction in synaptic transmission, which is a known characteristic of stress-induced hippocampal morphologic changes (Sousa and Almeida, 2002). In fact, stress was reported to suppress LTP in the hippocampal formation (Kim and Diamond, 2002), PFC (Rocher et al., 2004) and hippocampal-PFC connection (Cerqueira et al., 2007), but to facilitate LTP in the amygdala (Diamond et al., 2004). The hippocampal formation and PFC are involved in the integration of information and in working memory (Laroche et al., 2000), whereas the amygdala, which is connected with both the hippocampal formation and PFC, is related with processing of emotional memories (LeDoux et al., 2000). Therefore, these apparent paradoxical stress-induced

LTP actions may reflect the stress-associated deficits of hippocampal formation-PFC functions but also increased fear-related memories (Diamond et al., 2004). Importantly, these stress and corticosteroids induced functional and plastic alterations in these brain areas are related to cognitive and memory deficits (Sousa et al., 2000; McEwen et al., 2005; Cerqueira et al., 2007), which are known to be also present in depressive patients (Brown et al., 1999; Eastwood and Harrison, 2001).

Similarly to CMS and Dex, ADX also induced a decrease of BAG-1 and synapsin-I expression; however, contrary to the other experimental paradigms, ADX also decreased GSK-3β levels, probably as a result of the marked associated cell loss. In this condition there seems to be a possible association between decreased expression of BAG-1 and increased apoptosis, as well as between lower levels of GSK-3β and higher neurogenesis. These results support previous studies, which propose that MR activation is essential to the maintenance and function of anti-apoptotic pathways (Almeida et al., 2000). The ADX-induced decrease of GSK-3β also suggests that MR and GR basal activation contributes to the regulation of the expression of this enzyme (Chapter 2.3), as observed earlier (Sotiropoulos et al, 2006). Lithium administration to animals to whom the adrenals were removed (ADX+Li) restored synapsin-I levels and prevented BAG-1 decrease (Chapter 2.3), reinforce our previous observation that lithium neuroprotection may be independent of the regulation of corticosteroid milieu and occurs through a direct action on the above-mentioned molecular pathways.

Stress, corticosteroids and lithium are known also to influence the expression of growth factors, which significantly modulate apoptosis and neurogenesis. Besides regulating the birth of new neurons, some of these signalling molecules also act in the formation of new vessels (angiogenesis). Interestingly, post-natal neurogenesis occurs mainly in clusters associated with vascular niches (Palmer et al., 2000). Angiogenesis is a crucial event to the maintenance of these niches, and VEGF and its receptor Flk-1 are expressed by tissue around and by cells inside these clusters (Palmer et al., 2000). Hence, VEGF, as a potent angiogenic factor (Ferrara et al., 1997), might also influence neurogenesis. This was shown by Luissaint and colleagues (2002) which reported an interaction between angiogenesis and neurogenesis; furthermore, in this interplay, VEGF had an essential role. However, subsequently, it was observed that besides an indirect effect, VEGF also modulates neurogenesis directly (Jin et al., 2002). Therefore, we

thought of interest to analyse the effects lithium in VEGF hippocampal levels in basal and stress condition. In accordance with previous observations that stress- and Dex-induced a decrease of VEGF expression (Machein et la., 1999; Koedam et al., 2002; Heine et al., 2005; Wu et al., 2006), we also observed a decreased in VEGF hippocampal levels after CMS. This indicates that the distinct pathways through which CMS influence neurogenesis may also involve VEGF (Chapter 2.4). The impact of CMS in VEGF expression occurred in distinct cell populations known to express VEGF (immature neurons, mature neurons and astrocytes) (Meurer et al., 2003; Yang et al., 2003; Kutcher et al., 2004; Meng et al., 2006), indicating a wide-spectrum effect of CMS in VEGF-producing cells. Therefore, the CMS modulation of hippocampal dentate gyrus functions seems to occur not through the modulation of the activity of a specific cell type, but rather by acting in diverse cell families and at different stages of differentiation, suggesting that CMS influences mechanisms common to diverse cell lineages. Importantly, the mechanism through which CMS decreased VEGF levels seemed to occur involves the increase of GSK-3β, with a consequent decrease of β-catenin expression, which is in accordance with previous results (Orford et al., 1997; Lie et al., 2005) (Chapter 2.4).

Supporting the role of GSK-3β in CMS effects, lithium co-administration (CMS+Li) prevented all the CMS-induced effects, including the normalization of VEGF levels. This fits with the reported lithium effects in the ischemic myocardium (Kaga et al., 2006) and suggests that the neuroplastic actions of this drug also occur through VEGF modulation (Chapter 2.4). Additionally, VEGF, which is regulated by β-catenin (Skurk et al., 2005), seems to be an essential mediator of antidepressants actions (Warner-Schmidt and Duman, 2007). Hence, it seems that mood stabilizers and antidepressants display common pathways of actions, including the regulation of angiogenic factors, which are important in development, but also, in the maintenance of proper body function (Carmeliet et al., 2003). Moreover, these observations clarify the mechanisms through which these drugs influence mood.

Chapter 4

References

4. References

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Chapter 5

Final conclusions and Future perspectives

5. Final conclusions and Future perspectives

The work presented in this thesis confirmed and expanded the view that hippocampal neurogenesis is an adjustable cell event that is modulated by several factors. This flexibility endows the hippocampus with an enormous plasticity, which allows it to adapt and respond to environmental challenges. However, most factors influencing cell birth also modulate cell death, showing that neurogenesis is only one component of the complex program that determines cell turnover. Interestingly, hippocampal neurogenesis and apoptosis occur in tandem but do not display the same topographic maps, emphasizing the complexity of hippocampal structure and function. The hippocampal shifts in final cell balance, triggered by neuronal insults, were associated to changes in depressive behaviour. On the other hand, concomitant neuronal protection precluded the stress/glucocorticoids cellular and behavioural effects. In summary, we hope to have contributed to better understand the i) modulation of post-natal hippocampal neurogenesis and apoptosis; ii) molecular pathways through which these modulations occur; and iii) relevance of hippocampal cell balance in mood disorders, particularly in depression. These results may also disclose new potential therapeutic targets to the treatment of these devastating disorders.

However, the observations herein reported opened new questions. One of the most interesting results herein reported was the ability of lithium to prevent the stress/corticosteroid-induced effects. These results seemed to occur through lithium's action in GSK-3β. GSK-3β was previously proposed to modulate cell birth and death, synaptic plasticity, neurotransmitters, energy, and mood. In the demand for new therapeutics, this enzyme, due to its actions in several cellular mechanisms, was recently proposed as a possible new target in the treatment of depression. However, lithium has several other cellular targets, whose involvement could not be excluded by the present studies. Therefore it seems crucial to assess the importance of GSK-3β to the stress and lithium-induced changes in behaviour and hippocampal final cell balance. For that, additional studies would be necessary with a GSK-3β specific inhibitor. We propose to further investigate the effects of AR-A014418, a known specific GSK-3β inhibitor that has antidepressant effects, to animals concomitantly subjected to the CMS protocol. GSK-3β levels and other proteins that regulate neurogenesis and apoptosis are known to be increased by corticosteroids. However, as reported here, hypercortisolemia is not sufficient to induce a full

depressive-like behaviour, indicating the involvement of other concomitant events. Hence, and considering that CRF antagonists were reported to have antidepressant effects, it would also be very interesting to scrutinize the role of neurotransmitters (such as CRF and vasopressin) in hippocampal cell turnover. The assessment of a depressive-like behaviour would allow to clarify the contribution of alterations in the levels of these neurotransmitters in the etiology of depression. The study of changes in cell fate and associated molecular pathways could unveil some mechanisms through which these neurotransmitters would induce their effects. Taking into account that there is an alteration of depression clinical symtoms, corticosteroids milieu, neurogenesis and hippocampal function with aging, the question if the alterations here reported would also occur in aged rats is also appealing. This would allow to better understand the influence of altered hippocampal formation plasticity in the progression of depression with age. In summary, there are many questions and enigmas still to resolve; indeed, if it was necessary millenniums to build our brain, perhaps it will be required as much to understand how it works in health and disease.

Chapter 6

Attachements

Bessa J, Mesquita R, Silva R, Franky, A, Silva A, Cerqueira JJ, Sousa N. "Is the “Neurogenic Hypothesis” of Depression a Myth? New Insights from the Bench to the Bedside”. Abstract to the American Society Association annual meeting (2007)

Abstract

Depression is amongst the most prevalent psychiatric disorders. Recent findings concerning the pathophysiology of this disease suggest that hippocampal adult neurogenesis plays a critical role in the action of antidepressant drugs, giving rise to a "neurogenic hypothesis" of depression. To further understand these phenomena, this study examined the effects of antidepressive drugs in animals exposed to a classical model of depression, in the presence or absence of the alkylating agent methylazoxymethanol (MAM) used to prevent the proliferation of neuroblasts. Fluoxetine (10mg/kg), Imipramine (10mg/kg) and vehicle (saline) were chronically administered alone or with MAM (7mg/kg), to male Wistar rats in the last two weeks of exposure to a Chronic Mild Stress (CMS) protocol of eight weeks. Depressive-like behaviour was assessed with the forced swimming test (FST). Hippocampal cell proliferation and neurogenesis were assessed with immunocytochemical detection of BrdU incorporation and co-localization with neuronal markers. In the FST after exposure to CMS and drug treatment, immobility time was increased and latency to immobility decreased in the saline-treated group. Both Fluoxetine and Imipramine treatment were able to revert these behavioural effects of CMS in the FST confirming their antidepressant effect in this animal model. The concomitant use of MAM with Fluoxetine, Imipramine or saline did not alter the behavioural profile in any experimental group. Moreover, CMS let to a decreased neurogenesis in the hippocampus whereas the use of Fluoxetine and Imipramine increased cell proliferation in this region; as expected, the use of MAM significantly reduced cell proliferation in all therapeutic groups. In conclusion, these results suggest that neurogenesis is impaired in an animal model of depression (CMS) and is influenced by antidepressants. However, increased neurogenesis is not a necessary condition for the therapeutic effect of these drugs, thus calling for a re-appraisal of the role of hippocampal neurogenesis in depression.